Testing traditional concepts: biodiversity and integrative taxonomy of Brazilian opisthobranchs (Mollusca, Heterobranchia)

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1. SUMMARY

The knowledge on biodiversity and its maintenance is important as it has direct relation to human well-being. The basic unit for studies on biodiversity, and any related subjects, are species. Therefore, effective species-level taxonomy is fundamental for correct species delimitation and (re)identification. Brazil is considered the most biodiverse country of the world, mainly due its high number of inland species, while its marine biodiversity is comparatively less known. Several species-rich and abundant marine groups lack any specialist researchers. Sea slugs (traditionally grouped under the 'Opisthobranchia') represent a non-monophyletic, species rich group with remarkable, diverse and at the same time convergent morphologies and life styles, occurring in all kinds of marine and intertidal environments. Differently to most of the other, shell bearing, marine gastropods, for which taxonomy is strongly based in sculptural differences and proportions of hard shells, the taxonomy of opisthobranchs containing sea slugs and snails with reduced shells, is mainly or entirely based on soft body morphology. Species descriptions of Brazilian opisthobranchs usually were based on single or few specimens, collected from the intertidal or shallow subtidal. Descriptions included few features either from living specimens or from preserved, contracted and color faded specimens, turning comparisons and later reidentification a difficult task. The Brazilian opisthobranch fauna clearly needs revision, and its diversity is potentially underestimated.

The present thesis thus explores the opisthobranch diversity of Brazil and adjacent, rarely sampled regions, such as Atlantic islands, and habitats, including subtidal Scuba diving depths. Modern, integrative taxonomic approaches including molecular, histology-based and standard morphological techniques were applied to several example groups from different opisthobranch taxa. The exploration of previously rarely surveyed regions, such as Brazilian northeastern and southeastern coasts (chapters 1 and 2), and habitats revealed an unknown biodiversity, including undescribed species of different groups (chapters 4, 7 and 8), and permits a better assement of species biogeography. New data allowed, for example, the evaluation of the geographic distribution of some species along the tropical western Atlantic region. *Navanax gemmatus* (Mörch, 1863) (chapter 6) is distributed from the Caribbean Sea to Brazil, across the influence of the Amazon River outflow, while *Pleurobranchus areolatus* comprehends two distinct, not close related species, in these regions (chapter 9).

Newly collected specimens in Brazil usually showed an unexpected morphological variation and patterns that are in conflict with the available literature. In at least nine cases studied, competing hypotheses on intraspecific variability versus unrecognized species complexes were assessed by applying molecular phylogenetic and a range of species delimitation analyses. In seven of the nine cases, there is strong evidence for cryptic species, some of which were described as new species (e.g. *Cratena minor* Padula, Araújo, Matthews-Cascon & Schrödl, 2014; chapter 7).

This study indicates the limits of current Brazilian opisthobranch taxonomy and proposes a new taxonomic approach for the study of non-microscopic opisthobranchs in general. Starting with a molecular approach and a first step of barcoding at least one informative loci. Additional markers were included and alignments of the individual genes were the basis for molecular phylogenetic analyses and different species delimitation methods currently available. In addition, alignments were used to search for diagnostic molecular characters, which represent a simple way to indicate the molecular differences between species and, given a suitable sampling, to infer on molecular apomorphies. Initial molecular evidences where compared to morphological, color patterns and biological/ecological evidences, considering also the geographic distribution of the different delimited groups. This is resumed in a cost-effective, integrative taxonomic workflow applicable also to other non-microscopic, coastal and not necessarily abundant marine invertebrates.

Available schemes usually integrate taxonomic evidence either by accumulating evidence or by using a (minimum) congruence approach. However, in Brazilian opisthobranchs, the exact number of separate evolutionary lineages could not always been resolved, because of conflicting results according to different markers and analyses used. According to different quantities and quality of data and analyses, not only the numbers of supporting or compatible results but also the power of individual evidences need to be considered. Conflicting rather than fully supportive or compatible lines of evidence are a common problem in opisthobranch species delimitation, and taxonomic decisions are often intransparent. Therefore, an initial, still simple way of visualizing the historical and actual evidences for different hypotheses is proposed.

For all nine taxonomic cases studied herein, the number of species resulting from the (multi-evidenced) integrative taxonomic approach differs to the number of species delimited by traditional opisthobranch taxonomy. In general, species diversity is higher than previously believed, due the existence of cryptic diversity. In some cases however,

representatives of different species delimited through traditional taxonomy were confirmed to be part of a same species and traditional diagnostic characteristics observed to correspond to intraspecific variation. A remarkable case is the *Felimida clenchi* group. For the first time, extreme color polymorphism is observed and supported in brightly colored opisthobranch species, indicating that body color pattern is not a strict reliable taxonomic character as previously though. In addition to circular mimetic color groups, polychromatism is an interesting subject for further studies on the origem of the diversity color patterns in sea slugs.

2. INTRODUCTION

2.1 General introduction

Biodiversity contributes directly, by means of biological products, and indirectly, through environmental services, to human well-being, making the knowledge and biodiversity conservation priority activities in the current. Correct species identification is essential for any subsequent work, as ecological approaches, chemical prospection seeking for pharmaceutical compounds, and general studies on environment conservation. Brazil is the most biodiverse country of the world, mostly due its high number of inland species, such as plants, insects, birds and amphibians (Lewinsohn & Prado 2004, 2005). While inland biodiversity and ecosystem dynamics are somehow well known in Brazil, Brazilian marine diversity and ecosystems, in comparison, still await more comprehensive studies (Vilar et al. 2015). Not only Brazil, but many areas of the Atlantic Ocean, including Caribbean regions, isolated oceanic islands and most of the coast of Africa, have never been deeply studied concerning their marine species biodiversity, including genetics. In addition to little research directed to marine biodiversity in such regions, one of the current difficulties in the study of biodiversity is the taxonomic impediment, which means the incapacity of preparing descriptions of new species in the same rate as they are discovered (De Carvalho et al. 2007, 2008; Padial et al. 2010). The reduced number or absence of specialists in a neglected discipline, alpha taxonomy, is one of the main causes to the taxonomic impediment (Wägele et al. 2011, Johnson 2012). When compared to other countries, Brazil is a kind of exception in still forming, though in small numbers, new taxonomists and researchers in biodiversity. Many papers were published in recent years on the diversity of reef fish (e.g. Pinheiro et al. 2015), sponges (e.g. Bispo et al. 2014, Lopes & Hajdu, 2014), bryozoans (e.g. Vieira et al. 2014), among others. However, for many diverse and abundant groups, especially invertebrates, there are no taxonomic specialists. In particular the traditional morphology-based taxonomy is not generally complemented yet by modern, diverse and informative techniques, using e.g. molecular data and detailed 3D microanatomical reconstructions. There is broad consensus that taxonomy should be integrative, though opinions may vary on which analyses to be used and on how various lines of evidences should be integrated (Dayrat 2005, Padial et al. 2010, Miralles et al. 2011, Jörger & Schrödl 2013).

2.2 Opisthobranch molluscs

With approximately 130.000 described species and about 70.000 fossils, molluscs represent the second largest animal phylum (Haszprunar et al. 2008). Many species have economic importance, being used for human consumption, production of cultural value of artifacts, as bio-indicators of environmental quality, while others have pharmacological and public health importance. Among molluscs, the Gastropoda is the most diverse group with more than 100.000 described species (Aktipis et al. 2008). Gastropods were traditionally divided into three main groups: Prosobranchia, Pulmonata and Opisthobranchia (Milne Edwards, 1848, Thiele, 1929). The last two groups are part of the Euthyneura Spengel, 1881, which, together with the Allogastropoda, comprise the Heterobranchia (Haszprunar 1985, 1988). On one hand, the morphology-based concept of Heterobranchia was supported by cladistic analyses (Ponder & Lindberg 1997) and also in different recent molecular studies (Dinapoli & Klussmann-Kolb 2010, Dayrat et al. 2011, Jörger et al. 2010, 2014a, Schrödl et al. 2011). On the other hand, Opisthobranchia and Pulmonata were not supported in morphological phylogenetic studies (Haszprunar 1985, Dayrat & Tillier 2002).

Early molecular phylogenetic studies on the Euthyneura typically focused on either opisthobranchs or pulmonates. These studies resulted in different, contradicting hypotheses, most of them rejecting the monophyly of both groups (Dayrat & Tillier 2002, Grande et al. 2004a,b, Vonnemann et al. 2005, Wägele & Klussmann- Kolb 2005, Klussmann-Kolb et al. 2008). Some more recent studies contributed with a more comprehensive sampling of both opisthobranchs and pulmonates, including previously neglected taxa, such as the meiofaunal Acochlidia, and some 'lower heterobranchs' (allogastropods), as for example the Murchisonellidae (Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010, Schrödl et al. 2011, Brenzinger et al. 2013a, Wägele et al. 2013). The study of Jörger et al. (2010), based on phylogenetic analyses of multi-locus markers, convincingly rejected the monophyly of Opisthobranchia and Pulmonata, and proposes a new subdivision of the Euthyneura in Euopisthobranchia, Panpulmonata and Eupulmonata. This hypothesis was confirmed and complemented in subsequent works (Brenzinger et al. 2013, Wägele et al. 2014 Jörger et al. 2014), including two studies based on massive phylogenomic data (Kocot et al. 2013, Zapata et al. 2014). These recent evidences indicate that some characteristics shared by some euthyneuran groups, such as the loss of the shell and the switch to herbivory, evolved several times independently. Shelled forms, for example, are not obligatorily basal lineages among the Euthyneura; neither does the Nudibranchia, a shell less group, represent a derived lineage as traditionally believed (Schrödl, 2014).
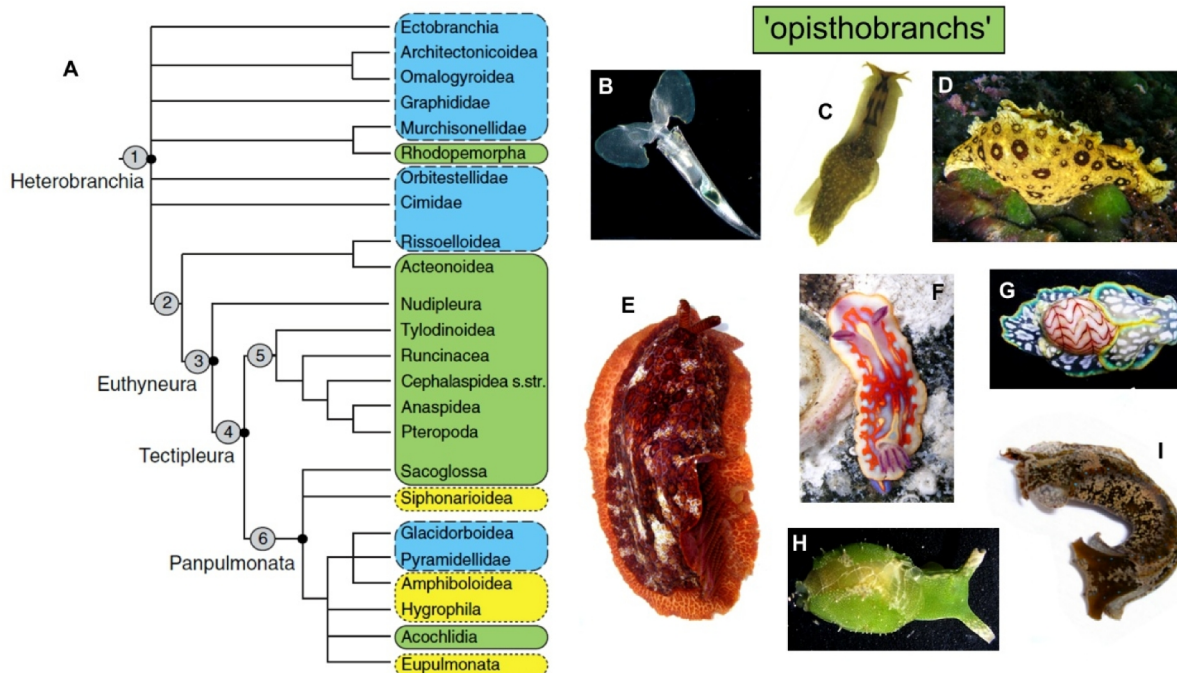
Figure 1. Phylogeny and diversity of 'Opisthobranchia'. A. Recent hypothesis on the phylogeny of the Heterobranchia (adapted from Wägele et al. 2014), traditional opisthobranch groups demarcated in green. B. Planktonic pteropod *Creseis virgula* (source: Census of Marine Zooplankton), C. Freshwater acochlidia *Acochlidium fijiiensis* (adapted from Jörger et al. 2010), D. Anaspidea *Aplysia dactylomela*, E. Nudipleura *Pleurobranchus reticulates*, F. Nudipleura *Felimida clenchi*, G. Acteonoidea *Micromelo undatus*, H. Sacoglossa *Oxynoe antillarum*, I. Cephalaspidea *Navanax gemmatus* (photos D-I by the author).

In total, the diversity of traditional 'opisthobranchs', a grade including marine slugs and their shelled relatives, is estimated in between 5.000 and 6.000 described species (Wägele & Klussman-Kolb 2005). Of these, roughly 3.000 belong to the Nudibranchia (Wägele & Willan 2000), which are the "sea slugs" in a strict sense. Most opisthobranch species are known from the tropical Indo-Pacific region where, however, many undescribed species are still being discovered (Gosliner et al. 2008). Some regions, such as the tropical Eastern Pacific and the Mediterranean Sea, have been studied for a long time and their opisthobranch fauna is relatively well known (MacFarland 1909, Cervera et al. 2004, Camacho-García et al. 2005; Behrens & Hermosillo 2005), but even for such areas new records and new species are still being recorded (Angulo-Campillo & Bertsch 2013, Prkic et al. 2014, Poursanidis & Koutsoubas 2015). Comparatively, other regions of the world were less studied, as is the case of the west and east coasts of South America, including the Brazilian coast; and other regions of the South Atlantic Ocean, including its oceanic islands.

2.3 Brazilian opisthobranch molluscs

The study of opisthobranch diversity in Brazil includes three main periods, the first with international expeditions along the Brazilian coast; the second with the large contribution of the German couple Ernst and Eveline Marcus and; third, with more recent contributions by Brazilian and foreign researchers (Padula et al. 2007). The first species recorded from Brazil were discovered during different North American and European expeditions between 1825 and 1915 (Padula et al. 2007). Among the first records, *Phylliroe lichtensteini* (Eschscholtz 1825), a pelagic nudibranch species originally described from Espírito Santo region and which is considered cosmopolitan nowadays. In 1852, *Marionia cucullata* (Couthouy 1852) was described based on specimens found among shrimps in a market in Rio de Janeiro. The famous 'Challenger Expedition' resulted in the collection of different species in 1873. Further contributions by Bergh (1878, 1879), Ihering (1886) and MacFarland (1909) resulted in the list of species published by Ihering (1915), covering a total of 34 opisthobranch species.

Between 1915 and 1952 no work on Brazilian opisthobranchs was published. The research on Brazilian species returned by the hands of Ernst and Eveline Marcus, who went to Brazil eloping from the Second World War (Mendes, 1994). Ernst, descendent of Jews and zoologist, got a position in the University of São Paulo, where he dedicated efforts not only studying molluscs but other marine invertebrate groups, such as bryozoans and polyclad flatworms (Mendes 1994). In his first comprehensive publication on Brazilian opisthobranchs, 32 species were examined, 21 of them new to science (Marcus 1955). In a publication two years later, other 17 new species were described (Marcus 1957). After the death of Ernst Marcus, in June of 1968 (Mendes 1994), his wife Eveline Marcus kept on exploring Brazilian and tropical western Atlantic opisthobranchs. In 1977, her annotated checklist on tropical Western Atlantic opisthobranchs included at least 155 species as occurring in Brazil (Marcus, 1977). Eveline Marcus continued publishing until her death, in 1990. During 37 years (1953-1990), Ernst and Eveline Marcus made the most substantial contribution on the knowledge of Brazilian opisthobranch diversity, reporting around two thirds of the species currently known from Brazil. Their tremendous historical contribution comes along with some problematic aspects: not all of their species descriptions were detailed enough to allow for secure re-identification, often lacking any information on living specimens; highly unfortunate for later research was the absence of designation and destination of voucher specimens, including of type-material, and the fact that the opisthobranch type collection was inherited privately rather than deposited in a public

museum. Thankfully to the effort of colleagues from the Museu de Zoologia da Universidade de São Paulo, partial collection of specimens studied by Marcus & Marcus was located and recovered from previous poor conditions and is now integrated as part of the malacological collection of the museum (Dornelas & Simone 2011, CM Cunha & LRL Simone pers. comm.)

The era of Ernst and Eveline Marcus, unfortunately, has not led to the development of local taxonomists for the study of Brazilian opisthobranch fauna. Only some isolated publications by different authors were done. Rios (1994), in his book on Brazilian marine molluscs, included a compilation of all species recorded at that time. More recently, works by Spanish authors, or partnerships between Spanish and Brazilian authors, resulted in taxonomic contributions on the genera *Nanuca* Marcus, 1957, *Phidiana* Gray, 1850, *Felimida* Ev. Marcus, 1971, *Felimare* Ev. Marcus & Er. Marcus, 1967 and *Tambja* Burn, 1962, including the description of some new species (Ortea et al. 1994, Troncoso, García & Urgorri 1998, García et al. 2002, García & Troncoso 2003, 2004, Pola et al. 2005). An exception from taxonomic works was the publication of two papers on chemistry, concerning the natural products of two nudibranch species. In these papers, however, the species studied were erroneously identified (Granato et al. 2000, 2005).

My contributions started with the first record of *Babakina anadoni* (Ortea, 1979) (under the name *B. festiva*) from Brazil, during my bachelor studies (Padula & Absalão 2005). One year later, we reported three additional new records (Padula & Santos 2006) from Brazil: *Aeolidiella alba* Risbec, 1928; *Berghia creutzbergi* Er. Marcus & Ev. Marcus, 1970 and *Flabellina engeli* Marcus & Marcus, 1968. The cases of *B. festiva* and *A. alba* were emblematic because both species were originally described from the Pacific Ocean, the last species being considered cosmopolitan, as well as many other opisthobranch species. Although this kind of geographic distribution was commonly accepted by the scientific community in that time, without deep discussion, this question caught my attention and I suggested that further studies should be done, including more accurate anatomical studies and molecular approach to confirm the identification of species with unexpected, wide, geographic distribution (Padula & Absalão 2005, Padula & Santos 2006).

Between 2007 and the start of this PhD thesis work in 2011, other contributions increased the knowledge on Brazilian opisthobranchs. DaCosta et al. (2007) proposed a new nudibranch subspecies, *Flabellina engeli lucianae* DaCosta, Cunha, Simone & Schrödl, 2007, in a study using advanced computer-based 3D anatomical reconstruction.

Among the papers resulteing from the 2nd International Workshop on Opisthobranchia, in Bonn 2006, two addressed Brazilian species, one including the redescription of the acochlid *Pontohedyle brasilensis* (Rankin, 1979) from São Paulo (Jörger et al. 2007) and the second a biogeographic approach on Brazilian opisthobranchs, by García et al. (2007). One year later, I published a short contribution on the morphology of two sacoglossan species from Rio de Janeiro (Padula 2008) and Domínguez et al. (2008) produced a revision on the nudibranch species of the family Aeolidiidae, including the description of one new species. The book of García et al. (2008) entitled 'Opistobrânquios do Brasil' compiled all the taxonomic information published on Brazilian species published until that time. The book includes photos of some species and new local records, listing a total of 241 species from Brazil. But this number includes the taxonomically problematic Pteropoda and many potential synonyms. The diversity of Brazilian opisthobranchs continued being documented, with regional reports on species distributions (Meirelles et al. 2009) and the description of new nudibranch species by DaCosta et al. (2010), Padula & Delgado (2010) and Alvim et al. (2011).

2.4 Gaps in the knowledge

Considering that many works were published on the diversity of Brazilian opisthobranchs, is there any need to go further? If yes, in what direction? Despite of the long period and great taxonomic contribution by Ernst and Eveline Marcus, new opisthobranch species are still being discovered and described from Brazil (e.g. Pola et al. 2005, DaCosta et al. 2010, Padula & Delgado 2010, Alvim et al. 2011), including species from greater depths in the sublittoral zone, an habitat rarely explored before. Along the more than 7400 km of the Brazilian coastline, covering tropical and subtropical waters, the data available on opisthobranchs is restricted mostly to São Paulo and Rio de Janeiro region, in the southeastern coast (Marcus 1977, Padula & Santos 2006, García García et al. 2008), which represent just a very small portion of the Brazilian coast. Data from the northern and southern Brazilian regions are comparatively smaller or, for many areas, inexistent. This illustrates how Brazilian opisthobranchs biodiversity, concerning number of species and their geographic distribution, is far from being well known.

In general, as reported for other molluscs and other groups, the Brazilian marine fauna is considered highly similar to the fauna of the Caribbean Sea. The similarity between the Brazilian and the Caribbean opisthobranch fauna was initially observed and discussed by Ev. Marcus & Er. Marcus (1960) and Edmunds (1964), and later in a biogeographic

analysis including gastropods in general (Floeter & Soares-Gomes 1999). The same pattern was also recorded for echinoderms (Tiago & Ditadi 2001, Hendler et al. 2005), crustaceans (Human & Deloach 2002) and reef fish (Rocha 2003, Feitoza et al. 2005). However, the long distance and putative barriers, such as the Amazon River outflow, between the Caribbean Sea and the Brazilian coast raise doubts about the conspecificity of material from both regions. This hypothesis remains untested for western Atlantic opisthobranchs.

With few exceptions, the majority of works on Brazilian opisthobranchs consist in simple morphological, species description papers. Apart of two chemical studies (Granato et al. 2000, 2005), two histology based 3D reconstructions and redescriptions (DaCosta et al. 2007, Jörger et al. 2007) and some recent, more detailed species descriptions (e.g., DaCosta et al. 2010, Padula & Delgado 2010, Alvim et al. 2011), the data available for most species is based on few morphological characters of few individuals from a single or few locations. Therefore, for most Brazilian species identity is still tied only to generally poorly described external, radular and reproductive system characteristics, a usual pratice in opisthobranch taxonomy (García et al. 2008). Histological, genetic, biological or any other feature that could facilitate species identification and the perception of a clear boundary between similar taxa remains largely unknown. In consequence, questions relative to the morphology, the correct identification and the real geographic distribution of some Brazilian species arose, as highlighted by Padula & Absalão (2005) and Padula & Santos (2006). Would some initial species descriptions overlooked or misidentified relevant anatomical structures? Would the distance and the Amazon River outflow act as a factor for separating opisthobranch species along the tropical western Atlantic region? Could a species have an amphi-South American and amphi-atlantic geographic distribution?

Traditional taxonomic, i.e. morphology-based, concepts were recently challenged through gene sequence analyses (Wilson & Lee 2005, Pola et al. 2007, Jörger et al. 2012, Krug et al. 2013), supplemented by accurate anatomical and histological data, 3D-modelling and information on species biology and ecology (e.g. DaCosta et al. 2007, Neusser & Schrödl 2007). Gene-based research on Antarctic marine invertebrates, for example, has uncovered cryptic speciation is involved in almost all "species" studied, boosting the known diversity (Held 2003, Held & Wägele 2005, Wilson et al. 2009). Prior to this thesis, no integrative taxonomic research was done focused on Brazilian opisthobranch representatives. Also, with few exceptions (e.g. Malaquias & Reid 2008, Pola et al. 2009), Brazilian opisthobranch species generally have not been included in broader phylogenetic studies, such as the ones in family or genus level (e.g. Chan &

Gosliner 2007, Pola et al. 2007, Johnson & Gosliner 2012), nor have they been included into ecological studies such as spongivory by dorid nudibranchs, a compiled data by Rudman & Bergquist (2007) which resulted in hypotheses on the evolution of this group of nudibranchs.

2.5 Aims of the thesis

The aims of this thesis are: 1) explore undersampled or unsampled habitats, such as the rarely explored subtidal zone, and regions of the Brazilian coast and adjacent areas in the Atlantic Ocean in order to i) increase the knowledge of species biodiversity and their geographic distribution, ii) obtain material of putative species complexes, and evidences, for integrative taxonomic studies; 2) explore the power of 3D-microanatomy reconstruction in the study of a little known meiofaunal Brazilian opisthobranch species; 3) under the unified species concept, in which species hypotheses support is directly related to the number and relevance of the diverse supporting lines of evidence (de Queiroz 2005, 2007), apply integrative taxonomic approaches to Brazilian opisthobranchs. Various groups, in which traditional taxonomy (i.e. based generally on few morphological characters of few specimens) has potentially failed in the delimitation of species, are selected. Based on the different cases studied and some reported recently in the literature, I would like to i) present a series of preliminary evidences of potential failure of the traditional taxonomy of non-microscopic opisthobranchs; ii) propose a cost-effective, initial integrative taxonomic workflow for non-microscopic, shallow water opisthobranchs; iii) establish a general, illustrative method to resume the power and contribution of the different evidences and hypotheses, making taxonomic decisions more objective and, in particular, more transparent, facilitating thus a revision in the light of future data.

The Results section and the papers included are divided in three main parts: I. Faunal surveys and new biodiversity data; II. Computer-based 3D reconstruction of a meiofaunal Brazilian sea slug, and; III. Molecular phylogeny and integrative taxonomy.

Faunal surveys and new biodiversity data (Chapters 1-4): *Chapters 1 to 4* of the thesis are about new scientific surveys in regions previously not well studied in the western and South Atlantic Ocean, such as the northeastern and southern Brazilian coasts, the Caribbean coast of Costa Rica and, for the first time, Ascension Island. These new collections help to fill several gaps in the knowledge of the diversity and geographic distribution of opisthobranchs in the western Atlantic, allow comparison to previously studied areas and provide specimens for further microanatomical and molecular studies (Chapters 5-11).

Computer-based 3D reconstruction of a meiofaunal Brazilian sea slug (Chapter 5):
Chapter 5 explores the microanatomy of the Brazilian meiofaunal *Pluscula cuica* Marcus, 1953, the only philinoglossan species described from the Americas and supposedly the most basal of the group, since it was described with characters that appear to be plesiomorphic and not found in the other genera. The microanatomical reconstruction allows the comparison to the original description and evaluation of some dubious features. It also explores several not usually considered organs as potential taxonomic characters.

Integrative taxonomy and molecular phylogeny (Chapters 6-11): *Chapters 6 and 7* cover two cases of potential species complexes. The first (Chapter 6) investigates *Navanax aenigmaticus* (Bergh, 1893), a supposedly amphi-South American and amphi-Atlantic species, with records from Peru, California, Brazilian coast and Ghana; the second (Chapter 7) evaluates the occurrence of *Cratena peregrina* (Gmelin, 1791), a common Mediterranean species, in the Brazilian coast. In both cases, an integrative taxonomic approach is used, considering evidences from gene trees from independent nuclear and mitochondrial markers, species delimitation analyses, and morphology. *Chapters 8, 9 and 10* present molecular phylogenetic hypotheses for a nudibranch family (Nembrothinae) and two nudipleuran genera (the dorid nudibranch *Felimida* and pleurobranchoidean *Pleurobranchus*), respectively, dealing also with species delimitation in problematic groups. Chapter 8 includes also the rediscovery and redescription of *Tambja divae* (Marcus, 1958), consisting the first record of the species after its original description, from southeastern Brazil. The paper still includes the description of five new species, delimited both by molecular data and morphology; two of them (*Tambja brasiliensis* and *Roboastra ernsti*) endemics from Brazil. Chapter 9 is a study on the systematics and biogeography of *Pleurobranchus* Cuvier, 1804, recognizing wide phenotypic plasticity and cryptic diversity among the different nominal species, including two species from Brazil. Chapter 10 includes the morphological redescription of a rare Mediterranean nudibranch species, *Felimida elegantula* (Philippi, 1844) within a molecular phylogeny including congeneric species from the same geographic region, and also some other species from the Pacific Ocean with similar color pattern. Chapter 10 does not include any Brazilian species, but the phylogeny hypothesis and the evaluation of the importance of the color pattern in the taxomomy of *Felimida* sets the scene for the research presented in the last chapter. Chapter 11 addresses a taxonomically problematic, putative complex of species of brightly colored opisthobranchs, the *Felimida clenchi* complex. Members of this group are distributed along the Atlantic, including the Caribbean Sea, Brazil and oceanic islands, such as Azores,

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Madeira and St. Helena, and also the Mediterranean Sea. This research tests the traditional , and rarely challenged, role of brightly colored body patterns as a primary character for nudibranch taxonomy. Molecular results obtained are unexpected, suggesting the existence of polychromatism within nudibranch species and existence of mimicry between closely and not closely related species.

3. RESULTS

Chapter 1

Padula V, Bahia J, Vargas C, Lindner A (2011) Mollusca, Nudibranchia: new records and southward range extensions in Santa Catarina, Southern Brazil. *Check List* **7**: 806-808.

Check List
Journal of species lists and distribution

Notes on Geographic Distribution

Mollusca, Nudibranchia: New records and southward range extensions in Santa Catarina, southern Brazil

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ABSTRACT: Nudibranch molluscs constitute a group of marine gastropods little studied in most of the Brazilian coast extension. Up to date, only ten species are known from Santa Catarina state, southern Brazil. This work presents four new records of nudibranchs from this region: *Aeolidiella indica* Bergh, 1988; *Berghia rissodominguezi* Muniain and Ortea, 1999; *Chromodoris paulomarcioi* Domínguez, García and Troncoso, 2006 and *Tambja stegosauriformis* Pola, Cervera, and Gosliner, 2005, expanding the known geographic distribution of the last two species more than 900 km southward.

The Santa Catarina state, southern Brazil (26–29° S), represents the southernmost limit of rocky shores in the tropical Southwest Atlantic (Floeter *et al.* 2008). Yet, the marginal reef sites in the region have only recently started to be studied in more detail, resulting in new records of tropical invertebrates (Rieger and Giraldi 1997) and particularly reef fishes (Barneche *et al.* 2009). In this study, we report four new records of nudibranchs from Santa Catarina state (Figure 1).

Nudibranchs constitute marine gastropods that lost completely the shell in the adult stage and their defense is given mostly by toxic products obtained from their prey, as cnidarians and sponges (Behrens 2005). Currently, approximately 100 nudibranch species are reported from Brazil (DaCosta *et al.* 2010) while around 3000 are known worldwide (Wagële and Klussman-Kolb 2005). Most of the available data on Brazilian nudibranchs result from the studies of Ernst and Eveline Marcus done between the 1950 and 1980 decades, most of them at southeastern Brazil (*e.g.* Marcus 1955; 1957). Recent studies resulted in new records and the description of new species from this region (Padula and Santos 2006; García García *et al.* 2008; DaCosta *et al.* 2010, Alvim *et al.* 2011) while northern, northeastern and southern Brazilian coasts remain poorly studied.

Despite the lack of studies focused on nudibranchs in southern Brazil, two of the firstly species know from Brazil, *Armina muelleri* (Ihering, 1886) and *Thordisa ladislavii* (Ihering, 1886), were described based on material from Santa Catarina state (Ihering 1886). The same work reported *Doris verrucosa* Linnaeus, 1758 (as *Staudoris verrucosa*) and *Marionia cucullata* (Couthouy, 1852) (as *Tritonia cucullata*) from the region (Ihering 1886). After a gap of almost a century, Marcus (1977) listed *Dendrodoris krebsii* (Mörch, 1863) and only 25 years later two other nudibranch species have been reported for Santa Catarina: the aeolids *Dondice occidentalis* (Engel, 1823) by Wiggers and Magalhães (2003) and *Spurilla neapolitana* (Delle

Chiaje, 1823), by Pimpão and Magalhães (2004). In 2006, the dorid *Hypselodoris lajensis* Troncoso, García and Urgorri, 1998 was reported to the Arvoredo Marine Biological Reserve (Domínguez *et al.* 2006) and one year later, DaCosta *et al.* (2007) described the subspecies *Flabellina engeli lucianae*, with distribution from Rio de Janeiro to Santa Catarina. Finally, a recent checklist added *Polycera aurisula* Marcus, 1957 to the list of marine
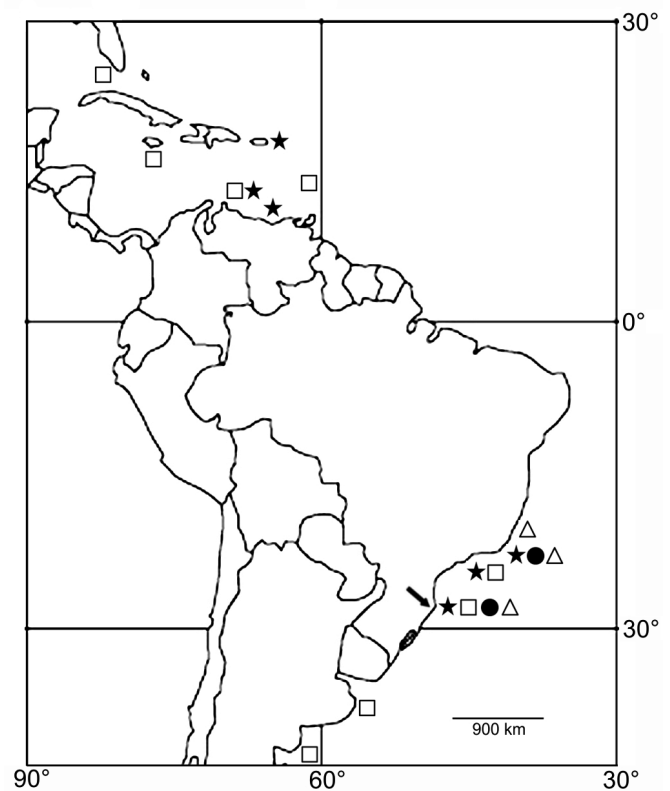

FIGURE 1. Geographic distribution of *Aeolidiella indica* (★), *Berghia rissodominguezi* (□), *Chromodoris paulomarcioi* (●), *Tambja stegosauriformis* (△) with indication of the news records from Santa Catarina state. *Aeolidiella indica* is a cosmopolitan species, records from the Pacific not indicated.

mollusks from Santa Catarina state (Agudo-Padrón *et al.* 2009), resulting in a total of 10 nudibranch species reported for the region up to date.

Due this scenario, the authors and collaborators conducted collections at Santa Catarina state, including the Arvoredo Marine Biological Reserve and adjacent areas under the permission 22583-1 of the ICMBio/SISBIO, Brazilian Ministry of Environment, with the objective to expand the knowledge on nudibranchs in this region. Material is deposited in the malacological collections of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ) and the Museu de Zoologia, Universidade de São Paulo (MZUSP). Collections at Praia da Armação (27°44'52.24" S, 48°29'55.93" W) resulted in two species previously unknown from southern Brazil: *Aeolidiella indica* Bergh, 1888 and *Berghia rissodominguezi* Muniain and Ortea, 1999. Two specimens of *A. indica* (Figure 2A) were collected intertidally, on 20 April 2007, by V. Padula and J. Bahia (MNRJ 11300; 8-17 mm long, alive). A single specimen of *B. rissodominguezi* (Figure 2B) was collected at the same locality and date (MZSP 96627; 15 mm long alive). *Aeolidiella indica* is a circumtropical species reported in Brazil from Rio de Janeiro and São Paulo states (Marcus and Marcus 1967; García García *et al.* 2008). *Berghia rissodominguezi* occur from Florida to Argentina and has been reported in Brazil at São Paulo state, as *Berghia coerulescens* by Marcus (1957) (see Muniain and Ortea 1999).

SCUBA divings at Arvoredo Marine Biological Reserve and adjacent areas, conducted in December 2009, resulted on the collection of other two species previously unknown from southern Brazil: *Chromodoris paulomarcioi*

Domínguez, García and Troncoso, 2006 and *Tambja stegosauriformis* Pola, Cervera and Gosliner, 2005. Up to now, *C. paulomarcioi* was only known from its type-locality at Búzios, Rio de Janeiro state, Brazil. A single specimen (Figure 2C) was collected at 6 m depth at Saco do Engenho (27°17'08" S, 48°22'13" W), 10 December 2009, by F. Azevedo and J. Carraro (MZSP 96626; 25 mm long preserved). *Chromodoris paulomarcioi* is very similar to the Caribbean species *Chromodoris grahami* Thompson, 1980, of which in fact may be a synonym. A comparative study is being conducted to clarify this question. The species *Tambja stegosauriformis*, previously known from Guarapari, Espírito Santo state (Rudman 2005) and the Cabo Frio region, Rio de Janeiro state (Pola *et al.* 2005), southeastern Brazil, was found at Ilha Deserta (27°16'22" S / 48°19'58" W). One specimen was collected (Figure 2D) at 12 m depth, 09 December 2009, by M. Kammers and L. Zago (MZSP 96625; 40 mm long preserved).

The present records of *Chromodoris paulomarcioi* and *Tambja stegosauriformis* expand their known geographic distribution more than 900 km southward (Figure 1). In addition, the distribution of some nudibranch species from the tropical Caribbean to southern Brazil, as *Dondice occidentalis* and *Berghia rissodominguezi*, the last one occurring also at the north of Argentina, suppose that they tolerate different environmental - water temperature and currents - and ecological conditions, as observed for some western Atlantic reef fishes (Barneche *et al.* 2009). However, the connectivity between Caribbean, northern and southern Brazil populations of nudibranchs was never investigated, representing an interesting subject for new studies.
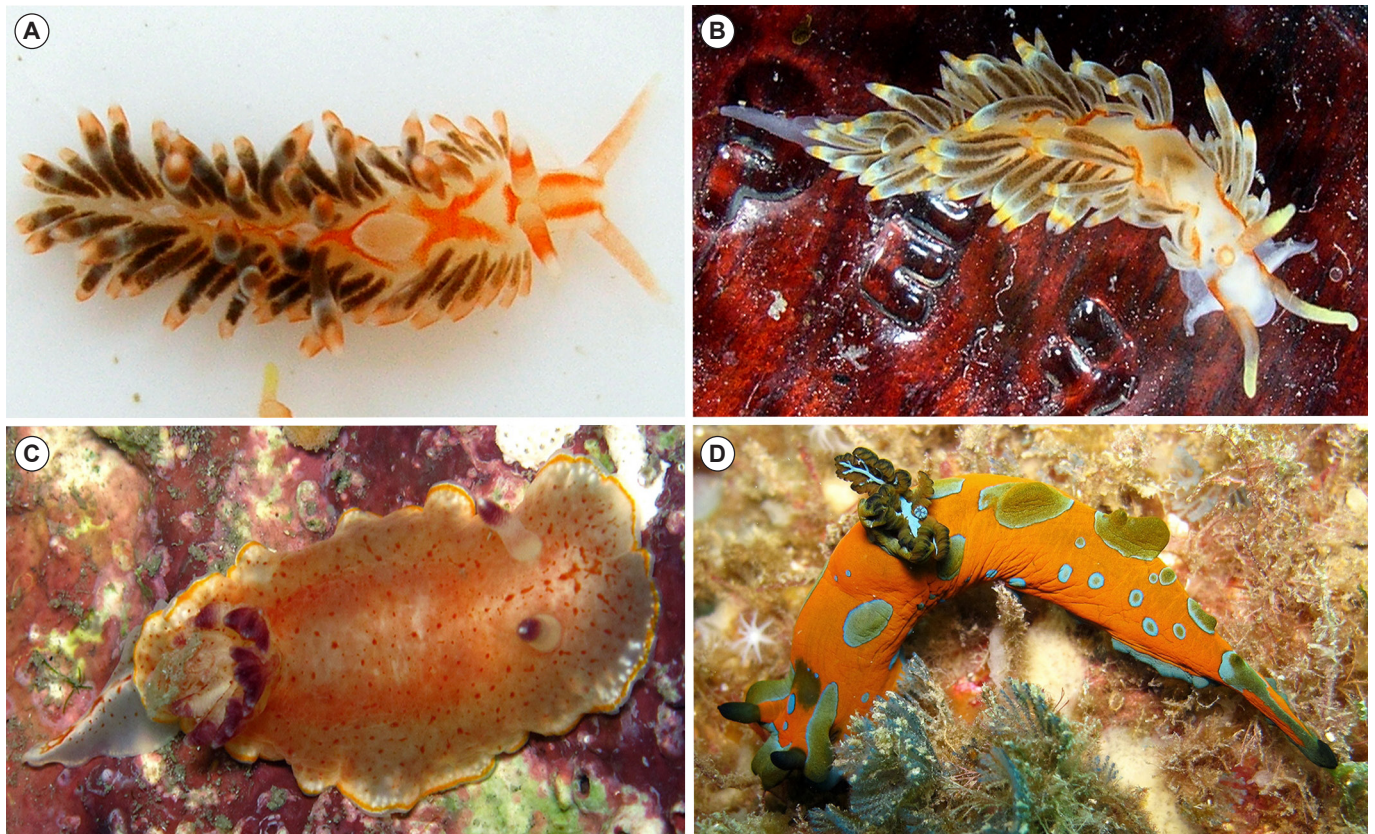
FIGURE 2. New nudibranch records from Santa Catarina state, southern Brazil. *A. Aeolidiella indica* from Praia da Armação (MNRJ 11300; 17 mm long alive); B: *Berghia rissodominguezi* from Praia da Armação (MZSP 96627; 15 mm long alive); C. *Chromodoris paulomarcioi* from Saco do Engenho (MZSP 96626; 25 mm long preserved); D. *Tambja stegosauriformis* from Ilha Deserta (MZSP 96625; 40 mm long preserved). Photos: A and B. Vinicius Padula; C. by João L.F. Carraro; D. by Leandro Zago.

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3. RESULTS

<u>Chapter 2</u>

Padula V, Bahia J, Correia MD, Sovierzoski HH (2012) New records of opisthobranchs (Mollusca: Gastropoda) from Alagoas, Northeastern Brazil. *Marine Biodiversity Records* **5**: e57.

New records of opisthobranchs (Mollusca: Gastropoda) from Alagoas, Northeastern Brazil

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The Brazilian Northeastern coast includes diverse ecosystems, particularly mangroves, coral and sandstone reefs. Although apparently high, the biodiversity of marine invertebrates from the State of Alagoas is still poorly documented. This study presents 28 new records of opisthobranch molluscs from Alagoas, 11 of which are also new records from the Brazilian Northeastern coast. In addition to comprising an important addition to knowledge about Alagoas reef biodiversity, the new records are useful data for future investigations such as biogeographical studies. Therefore, a list of all opisthobranch species known to date from Alagoas, as well as colour photographs of 32 species is provided.

INTRODUCTION

Opisthobranchs include marine and estuarine and a few limnic gastropod species, characterized by diverse body shapes, life habits and feeding specializations, with the shell reduced or completely lost in the adult stage of life. Traditionally considered a natural group within the Gastropoda, opisthobranchs are now understood to be polyphyletic (see Schrödl *et al.*, 2011).

Most species occur in tropical and subtropical shallow waters worldwide, but some are typical of cold and deep waters (Behrens, 2005). Most opisthobranchs belong to the carnivorous subgroup Nudibranchia (~3000 species), which completely lack a shell as adults (Wägele & Willan, 2000). Knowledge about Brazilian opisthobranchs is derived mostly from taxonomic studies by Ernst and Eveline Marcus, on material from the intertidal zone of the State of São Paulo and a few samples from
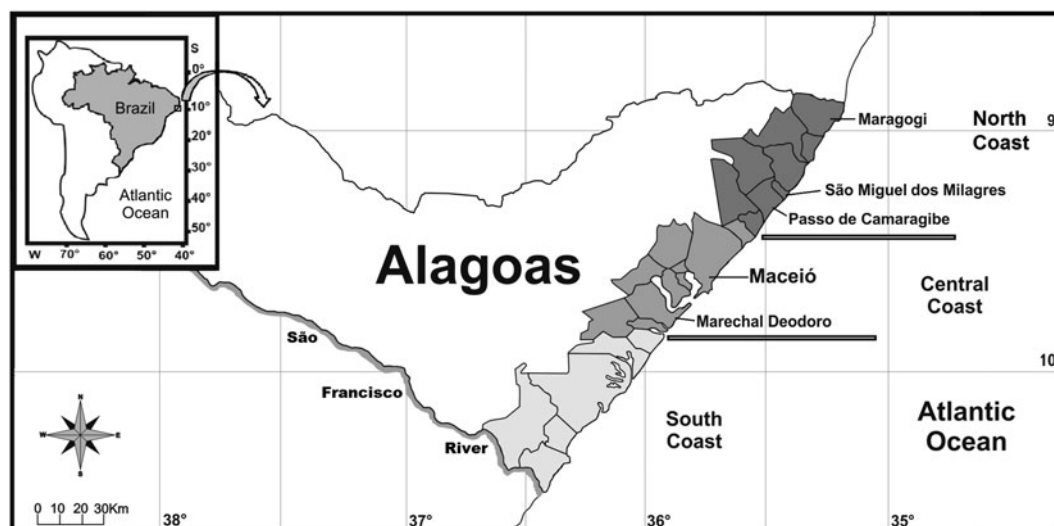
Fig. 1. Area of study: north and central coasts of Alagoas, Northeastern Brazil (adapted from Correia & Sovierzoski, 2009).

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Fig. 2. Reefs on the north coast of Alagoas, Brazil: (A) Maragogi–Gales coral reef; (B) São Miguel dos Milagres–Porto da Rua coral reef; (C) Passo de Camaragibe–Morro de Camaragibe coral reef.

Fig. 3. Reefs on the central coast of Alagoas, Brazil: (A) Maceió—1. Ponta do Prego coral reef and 2. Ponta do Meirim coral reef; (B) 3. Sereia sandstone reef and 4. Riacho Doce coral reef; (C) 5. Jatiúca coral reef, 6. Ponta Verde coral reef, 7. Piscina dos Amores coral reef, 8. Pajuçara coral reef and 9. Sobral pier; (D) Marechal Deodoro—10. Channel of the Manguaba lagoon, 11. Saco da Pedra sandstone reef and 12. Francês sandstone reef.

Table 1. New records, localities of collection and collection number of voucher specimens.

Taxon	Localities in Alagoas	Collection number
SACOGLOSSA		
Caliphylla mediterranea (A. Costa, 1867)	Ponta Verde and Pajuçara coral reefs (on the algae *Bryopsis* sp.)	MNRJ 13101
Elysia evelinae (Marcus, 1957)	Pajuçara coral reef (on the algae *Bryopsis* sp.)	Single specimen found donated to the colleague P. Krug
Elysia subornata (Verril, 1901)	Pajuçara coral reef (on the algae *Bryopsis* sp.)	MNRJ 18768
Elysia tuca (Marcus & Marcus, 1967)	Piscina dos Amores coral reef (on the algae *Halimeda* sp.)	MNRJ 18771
Oxynoe antillarum (Mörch, 1863)	Ponta Verde, Pajuçara and Piscina dos Amores coral reefs (on the algae *Caulerpa racemosa*)	MNRJ 12926, 12934
Polybranchia sp.	Sobral pier (on fouling community)	Material lost
APLYSIOMORPHA		
Bursatella leachii (Blainville, 1817)	Morros de Camaragibe and Manguaba lagoon estuary	MNRJ 12943
NUDIPLEURA		
PLEUROBRANCHOMORPHA		
Berthella sp.	Saco da Pedra sandstone reef	MZSP 97088
Pleurobranchus areolatus (Mörch, 1863)	Saco da Pedra sandstone reef	MNRJ 12928
Pleurobranchus atlanticus (Abbot, 1949)	Maragogi–Gales coral reef	MNRJ 18760
DORIDOIDEA		
Cadlina rumia (Marcus, 1955)	Ponta Verde and Pajuçara coral reefs; Saco da Pedra sandstone reef	MNRJ 12933
Chromodoris binza (Marcus & Marcus, 1963)	Maragogi–Gales coral reef; Saco da Pedra and Francês sandstone reefs	MNRJ 13100, MZSP 97077
Chromodoris paulomarcioi (Domínguez, García & Troncoso, 2006)	Saco da Pedra sandstone reef	MZSP 97078
Dendrodoris cf. *krebsii* (Mörch, 1863)	Riacho Doce coral reef; Saco da Pedra sandstone reef	MNRJ 12920, 12945
Geitodoris pusae (Er. Marcus, 1955)	Riacho Doce and Pajuçara coral reefs; Saco da Pedra and Francês sandstone reefs	MNRJ 13202
Hoplodoris hansrosarum (Domínguez, García & Troncoso, 2006)	Saco da Pedra sandstone reef	MNRJ 12924
Platydoris angustipes (Mörch, 1863)	Saco da Pedra sandstone reef	MNRJ 18762 (young specimen)
Rostanga byga (Er. Marcus, 1958)	Saco da Pedra sandstone reef	MZSP 97057
Taringa telopia (Marcus, 1955)	Saco da Pedra and Francês sandstone reefs	MNRJ 12923
Taringa sp.1	Pajuçara coral reef and Francês sandstone reefs	MNRJ 12922
Taringa sp.2	Saco da Pedra sandstone reef	MZSP 97058
AEOLIDIOIDEA		
Berghia rissodominguezi (Muniain & Ortea, 1999)	Sobral pier (on fouling community)	MNRJ 12925 (young specimen)
Eubranchus sp.	Manguaba lagoon	MNRJ 12951
Flabellina dushia (Marcus & Marcus, 1963)	Francês sandstone reef	MNRJ 18773
Flabellina engeli (Marcus & Marcus, 1968)	Saco da Pedra sandstone reef	Not collected
Glaucus atlanticus (Forster, 1777)	Francês sandstone reef	Not collected
Nanuca sebastiani (Marcus, 1957)	Sobral pier (on fouling community)	MNRJ 12944
Phidiana lynceus (Bergh, 1867)	Sobral pier (on fouling community); Saco da Pedra and Francês sandstone reefs	MNRJ 12921

other regions of the Brazilian coast (Marcus, 1955, 1957, 1971). The biodiversity of Brazilian opisthobranchs is clearly underestimated (Rios, 2009). Recently, sampling in new localities resulted in new country records (García & Troncoso, 2003; Padula & Absalão, 2005; Padula & Santos, 2006; García García et al., 2008; Sales et al., 2011) and the description of new species (DaCosta et al., 2010; Padula & Delgado, 2010; Alvim et al., 2011; Cunha, 2011). Currently, around 200 opisthobranch species, excluding the pelagic Thecosomata and Gymnosomata, are listed from Brazil (García García et al., 2008).

The Brazilian Northeastern coast includes a variety of ecosystems, particularly mangroves, coral and sandstone reefs. The reef ecosystems occur over a large area from the coast of Rio Grande do Norte State to the south coast of Alagoas, near the mouth of the São Francisco River (Castro & Pires, 2001). Although apparently high, the biodiversity of marine invertebrates from Alagoas is still poorly documented. Information is available for a few groups such as Porifera (Sarmento & Correia, 2002; Cedro et al., 2007, 2011); scleractinian corals (Correia, 2011), Bryozoa (Vieira et al., 2007, 2008, 2010) and Echinodermata (Lima et al., 2011). Currently, 22 opisthobranch species are known from Alagoas (Rios 1994, 2009; García García et al., 2008; pelagic Thecosomata and Gymnosomata excluded). Most records resulted from oceanographic expeditions (MacFarland, 1909) and others were obtained from isolated samples (Marcus, 1971).

This contribution presents new opisthobranch records from Alagoas, including groups that are traditionally included in 'Opisthobranchia': such as Sacoglossa and Nudipleura (Pleurobranchomorpha plus Nudibranchia). Material was obtained from a 10-day survey conducted by the authors and also from ecological studies carried out in recent years in the Setor de Comunidades Bentônicas (LABMAR/ICBS),
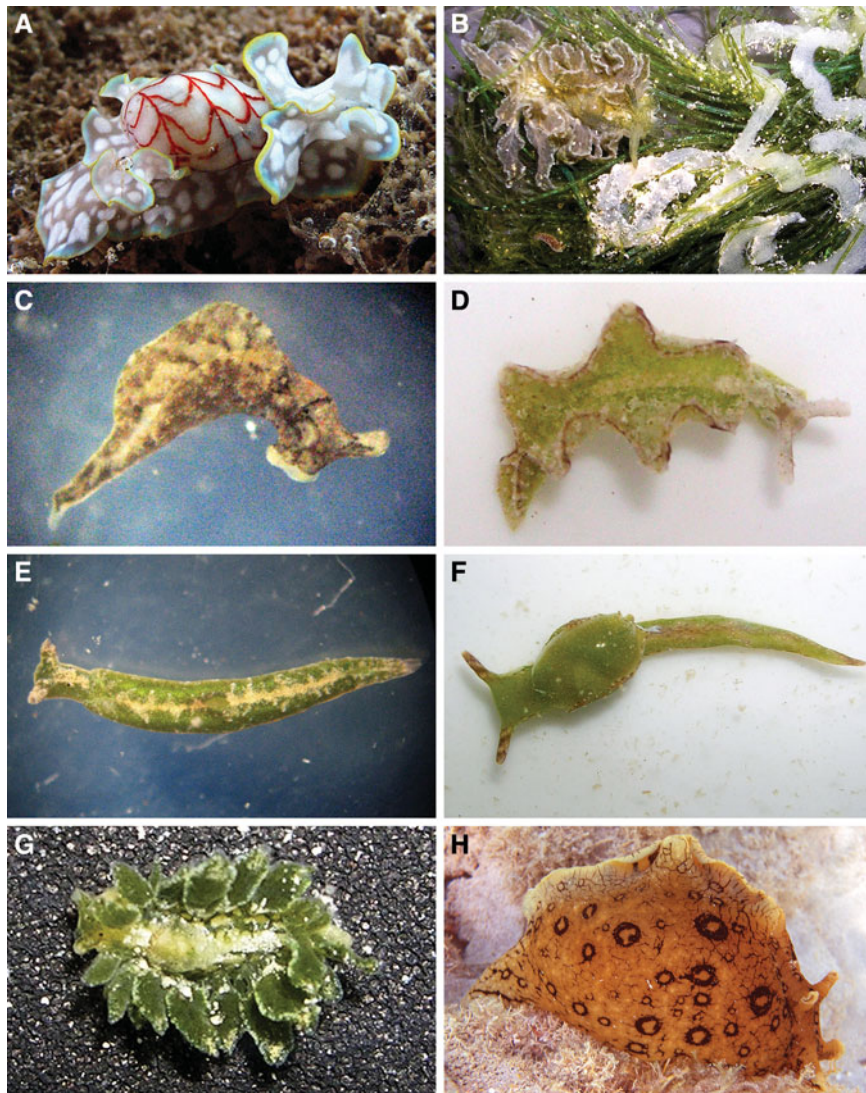
Fig. 4. Opisthobranchs from Alagoas: (A) *Micromelo undatus* (23 mm long); (B) *Caliphylla mediterranea* (14 mm long); (C) *Elysia evelinae* (4 mm long); (D) *Elysia subornata* (13 mm long); (E) *Elysia tuca* (9 mm long); (F) *Oxynoe antillarum* (13 mm long); (G) *Polybranchia* sp. (4 mm long); (H) *Aplysia dactylomela* (250 mm long).

Universidade Federal de Alagoas, Brazil. A list of all opisthobranch species known to date from Alagoas (pelagic Thecosomata and Gymnosomata excluded), and colour photographs of 32 species are provided.

MATERIALS AND METHODS

Study area

The coast of Alagoas is approximately 230 km long, bordered on the north by the Persinunga River and on the south by the São Francisco River (8°54′S–35°9′W and 10°30′S–36°23′W), and is composed mainly of coral and sandstone reefs, lagoons, rivers and mangrove ecosystems. The reef ecosystems of Alagoas originated from two main geological processes. The coral reefs were formed on calcareous sedimentary rock, composed of an aggregation of dead organisms, including skeletons of corals and hydrocorals combined with crusts of calcareous algae and other invertebrates. Many of these

fringing reefs are located near the beach line, where the top of the reef platform is exposed during low tides (Figure 2C). The sandstone reefs were formed by old sandbanks solidified through sedimentation, starting from chemical reactions with calcium carbonate from the Quaternary Period, and are generally arranged in rows parallel to the coastline and near the outlets of rivers and estuaries (Correia & Sovierzoski, 2009; Correia, 2011) (Figures 2–3).

The collection sites were located along the north and central coast of Alagoas, including (from north to south): Maragogi–Gales coral reef (9°01′07″S–35°12′13″W); São Miguel dos Milagres–Porto da Rua coral reef (9°15′11″S–35°20′31″W); Passo de Camaragibe–Morro de Camaragibe coral reef (9°20′40″S–35°26′54″W); Maceió–Ponta do Prego coral reef (9°31′48″S–35°35′30″W), Ponta do Meirim coral reef (9°32′37″S–35°36′52″W), Sereia sandstone reef (9°34′04″S–35°38′46″W), Riacho Doce coral reef (9°34′55″S–35°39′25″W), Jatiúca coral reef (9°39′12″S–35°41′46″W), Ponta Verde coral reef (9°39′57″S–35°41′32″W), Piscina dos Amores coral reef (9°40′39″S–35°42′10″W), Pajuçara
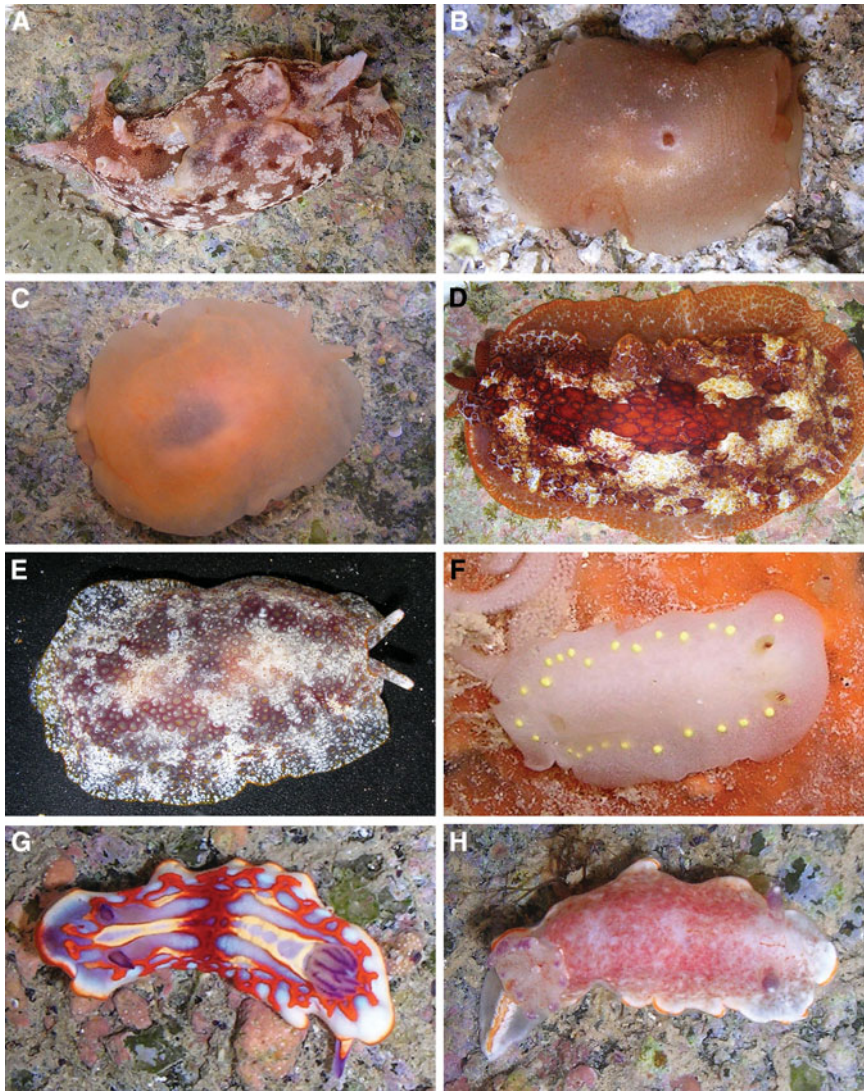
Fig. 5. Opisthobranchs from Alagoas: (A) *Aplysia cervina* (70 mm long); (B) *Berthella* sp. (13 mm long); (C) *Berthellina quadridens* (31 mm long); (D) *Pleurobranchus areolatus* (80 mm long); (E) *Pleurobranchus atlanticus* (27 mm long); (F) *Cadlina rumia* (8 mm long); (G) *Chromodoris binza* (14 mm long); (H) *Chromodoris paulomarcioi* (18 mm long).

coral reef (9°41′06″S–35°43′22″W) and Sobral pier (9°40′45″S–35°45′00″W); and Marechal Deodoro–channel of the Manguaba lagoon (9°43′13″S–35°48′23″W), Saco da Pedra sandstone reef (9°44′26″S–35°48′59″W) and Francês sandstone reef (9°46′03″S–35°50′13″W) (Figures 1–3).

Collection and material processing

Field observations and material collections were made through snorkelling in tide pools and reef edges during low tides. Samples of algae and fouling organisms were also collected to study the associated opisthobranch species. Aerial photographs were taken by M.D. Correia, and specimen photographs were taken by V. Padula. Some specimens were photographed *in situ*, with a Canon digital camera in an underwater Canon housing. Material was collected manually and stored in small plastic containers with seawater. In the laboratory, the specimens were measured and photographed, frozen in seawater, and then preserved in 70% ethanol. Some of the material obtained from ecological studies by

LABMAR/ICBS, found associated with macroalgae and sponges, was fixed in 4% formalin and preserved in 70% ethanol. Species identification was based on body colour and morphology, compared to original descriptions and field guides (Valdés *et al.*, 2006). The material is deposited in the malacological collections of the Setor de Comunidades Bentônicas/Universidade Federal de Alagoas (UFAL/MOL), Museu Nacional/Universidade Federal do Rio de Janeiro (MNRJ) and Museu de Zoologia da Universidade de São Paulo (MZSP).

RESULTS

Twenty-eight species representing new records from Alagoas were identified (Table 1; Figures 4–7). Eleven of the species also represent new records from the Brazilian Northeastern coast: Sacoglossa—*Elysia evelinae* Marcus, 1957 and *Elysia subornata* Verril, 1901; Doridoidea—*Chromodoris binza* Marcus & Marcus, 1963, *Chromodoris paulomarcioi*
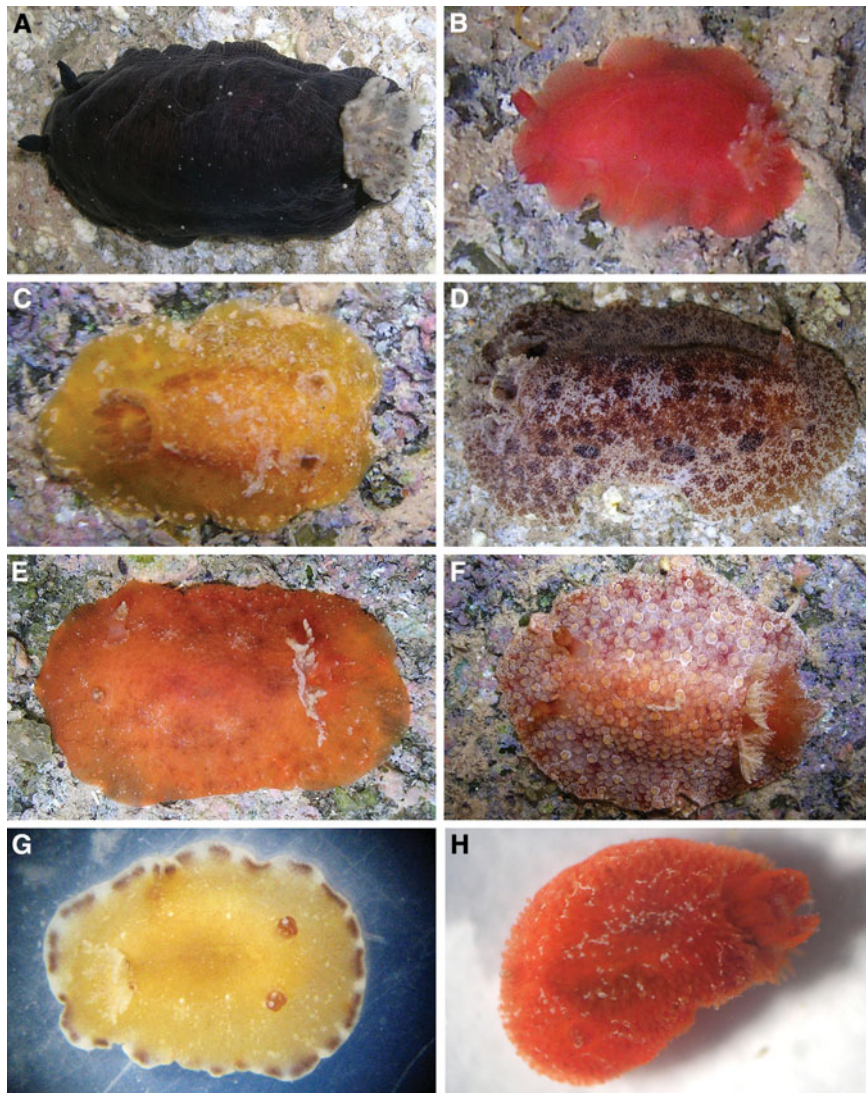
Fig. 6. Opisthobranchs from Alagoas: (A) *Dendrodoris* cf. *krebsii* (30 mm long); (B) *Dendrodoris* cf. *krebsii*, young specimen (9 mm long); (C) *Diaulula greeleyi* (14 mm long); (D) *Discodoris branneri* (46 mm long); (E) *Geitodoris pusae* (15 mm long); (F) *Hoplodoris hansrosarum* (28 mm long); (G) *Platydoris angustipes* (7 mm long); (H) *Rostanga byga* (5 mm long).

Domínguez, García & Troncoso 2006, *Dendrodoris* cf. *krebsii* (Mörch, 1863), *Geitodoris pusae* (Er. Marcus, 1955), *Hoplodoris hansrosarum* Domínguez, García & Troncoso, 2006, and *Taringa telopia* Marcus, 1955; and Aeolidioidea— *Berghia rissodominguezi* Muniain & Ortea, 1999, *Flabellina dushia* (Marcus & Marcus, 1963), and *Flabellina engeli* Marcus & Marcus, 1968 (see Table 2). This is the first record of *Hoplodoris hansrosarum* after its original description, which was based on material from Búzios, Rio de Janeiro, Southeastern Brazil (Domínguez *et al.*, 2006). The morphotypes *Berthella* sp., also known from Puerto Rico (Valdés *et al.*, 2006: 110), *Taringa* sp. 1 and *Taringa* sp. 2 probably represent undescribed species.

Most specimens were found under rocks and under reef fragments. Francês and, mainly, Saco da Pedra sandstone reefs were the richest collecting sites (Figure 3D). Arranged in rows parallel to the coast, these reefs have inner zones that are protected from waves. In shaded formations such as small caves in these protected zones, communities of sessile invertebrates such as cnidarians, sciaphilous sponges and tunicates develop. These invertebrates constitute the main food of the Nudipleura, the group with the most species collected in the present study (Table 1). The aplysiomorph *Bursatella leachii* Blainville, 1817 was common during summer months, when it was found in estuaries. Many individuals were observed associated with different macroalga species.

DISCUSSION

The 28 new records reported here (Table 1) comprise more than the total of 22 opisthobranch species that were previously known from Alagoas (Marcus, 1971; Rios, 1994, 2009; García García *et al.*, 2008). With the current records the total number of species increases to 50 (Table 2). This hidden biodiversity, now at least in part revealed, reflects the lack of specific research in the region, and illustrates the current state of knowledge for many marine invertebrate groups on the Brazilian coast: sparse information and gaps in the known geographical distributions. The 11 new records from the
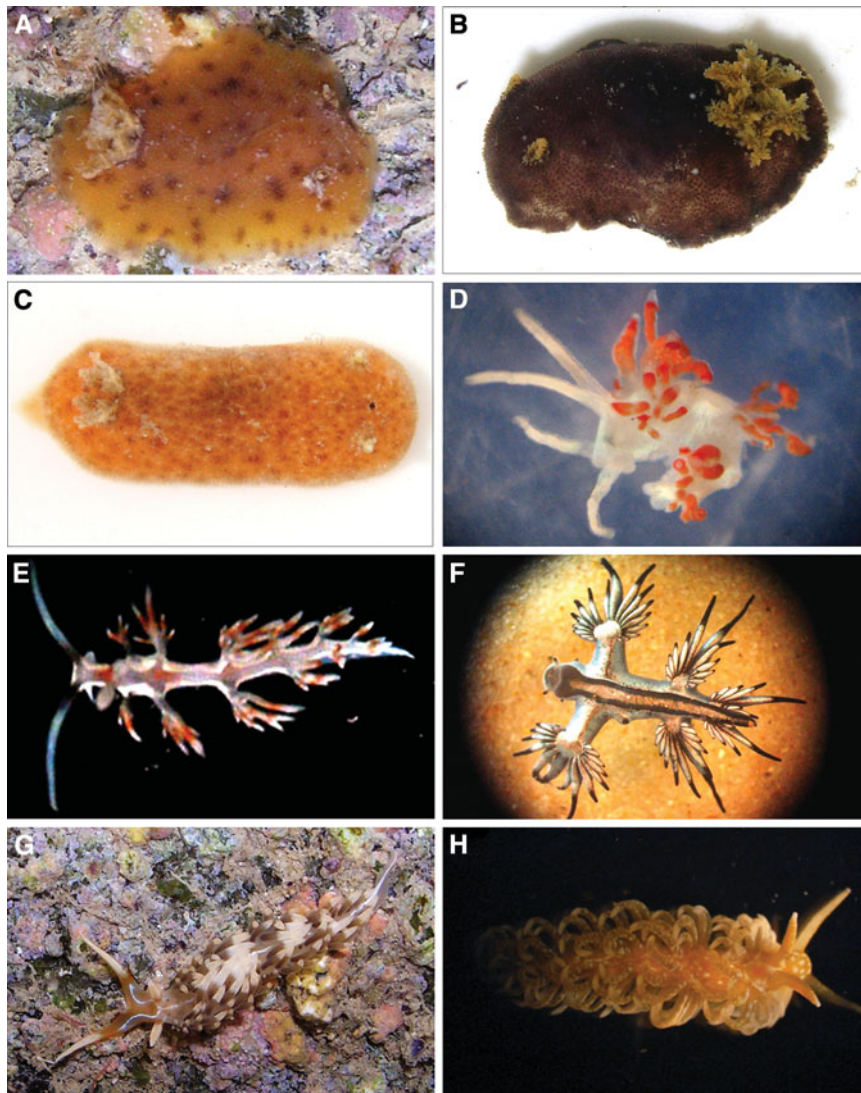
Fig. 7. Opisthobranchs from Alagoas: (A) *Taringa telopia* (11 mm long); (B) *Taringa* sp.1 (15 mm long); (C) *Taringa* sp.2 (7 mm long); (D) *Flabellina dushia* (3 mm long); (E) *Flabellina engeli* (12 mm long); (F) *Glaucus atlanticus* (18 mm long); (G) *Phidiana lynceus* (13 mm long); (H) *Spurilla neapolitana* (20 mm long).

Table 2. Opisthobranch species from Alagoas: their general geographical distribution and their distribution in Brazil.

Taxon	Geographical distribution	Distribution in Brazil	Source
ACTEONOIDEA			
Acteon danaida (Dall, 1881)	Florida, Gulf of Mexico, Brazil	Northeastern (Alagoas)	Dall, 1881; Marcus, 1971
Hydatina vesicaria (Lightfoot, 1786)	Bermuda, Florida to Brazil	Northeastern (Ceará, Pernambuco, Alagoas, Bahia)	Rios, 1994; García García *et al.*, 2008
Micromelo undatus (Bruguière, 1792)	Circumtropical	Northeastern (Pernambuco, Alagoas, Bahia)	Marcus & Marcus, 1967; García García *et al.*, 2008; present study
Toledonia vagabunda (Mabille, 1885)	Argentina, Brazil?	Northeastern (Alagoas)?	Marcus, 1971; Marcus, 1976
CEPHALASPIDEA			
Acteocina candei (d'Orbigny, 1841)	Bermuda, Bahamas, Florida to Brazil, Argentina	All coast	Rios, 1994; Valdés *et al.*, 2006
Atys caribaeus (d'Orbigny, 1841)	North Carolina, Florida to Brazil	Northern (Piauí); Northeastern (Alagoas)	Marcus, 1971; Rios, 1994; Valdés *et al.*, 2006
Atys macandrewii (E.A. Smith, 1872)	Gulf of Mexico, Caribbean Sea, Brazil; Azores, Canary Islands, Madeira	Northern (Maranhão); Northeastern (Alagoas)	Marcus, 1971; Cervera *et al.*, 2004; Valdés *et al.*, 2006
Bulla occidentalis (Adams, 1850)	From Bermuda to southern Uruguay	All coast	Dall, 1901; Malaquias & Reid, 2008

Continued

Table 2. Continued

Taxon	Geographical distribution	Distribution in Brazil	Source
Cylichnella bidentata (d'Orbigny, 1841)	From North Carolina to Brazil	All coast	Rios, 1994; Valdés *et al.*, 2006
Haminoea antillarum (d'Orbigny, 1841)	Bermuda, Florida to Brazil	Northeastern (Ceará, Alagoas, Bahia); Southeastern (São Paulo)	Rios, 1994; Valdés *et al.*, 2006
Haminoea elegans (Gray, 1825)	Bermuda, Florida to Brazil	All coast	Rios, 1994; Valdés *et al.*, 2006
Volvulella persimilis (Mörch, 1875)	Bermuda, Florida to Brazil	All coast	Rios, 1994
SACOGLOSSA			
Caliphylla mediterranea (A. Costa, 1867)	Florida, Caribbean Sea, Brazil; Mediterranean Sea, Northwestern Africa	Northeastern (Fernando de Noronha, Alagoas*); Southeastern (São Paulo)	Cervera *et al.*, 2004; Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Cylindrobulla beauii (P. Fischer, 1857)	Bermuda, Bahamas, Florida to Brazil	Northern (Piauí); Northeastern (Alagoas)	Rios, 1994; Valdés *et al.*, 2006
Elysia evelinae (Marcus, 1957)	Florida, Costa Rica, Brazil	Northeastern (Alagoas*); Southeastern (São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Elysia subornata (Verril, 1901)	Bermuda, Bahamas, Florida to Brazil; Madeira, Canary and Cape Verde Islands	Northeastern (Alagoas*); Southeastern (Rio de Janeiro, São Paulo,)	Padula, 2008; present study
Elysia tuca (Marcus & Marcus, 1967)	Bermuda, Florida to Brazil	Northeastern (Pernambuco, Alagoas*, Bahia)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Oxynoe antillarum (Mörch, 1863)	Bermuda, Bahamas, Florida to Brazil	Northeastern (Ceará, Pernambuco, Alagoas*, Bahia); Southeastern (Rio de Janeiro, São Paulo)	Valdés *et al.*, 2006; Padula, 2008; Meirelles *et al.*, 2010; present study
Polybranchia sp.	Brazil	Alagoas*	Present study
APLYSIOMORPHA			
Akera bayeri (Marcus & Marcus, 1967)	Florida, Colombia, Brazil	Northern (Piauí); Northeastern (Alagoas)	Marcus, 1971
Aplysia cervina (Dall & Simpson, 1901)	Florida to Brazil	Northeastern (Pernambuco, Alagoas)	MacFarland, 1909; Rios, 1994; present study
Aplysia dactylomela (Rang, 1828)	Circumtropical	Northern coast to São Paulo	MacFarland, 1909; Rios, 1994; present study
Bursatella leachii (Blainville, 1817)	Circumtropical	Northeastern (Pernambuco, Alagoas*); Southeastern (Rio de Janeiro, São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
NUDIPLEURA			
PLEUROBRANCHOMORPHA			
Berthella agassizi (MacFarland, 1909)	Bermuda, Caribbean Sea, Brazil	Northeastern (Pernambuco, Alagoas); Southeastern (Rio de Janeiro, São Paulo)	MacFarland, 1909; Valdés *et al.*, 2006; García García *et al.*, 2008; present sudy
Berthella sp.	Puerto Rico, Brazil	Alagoas*	Valdés *et al.*, 2006; present study
Berthellina quadridens (Mörch, 1863)	Caribbean Sea, Brazil	Northeastern (Alagoas, Bahia)	Marcus & Marcus, 1969; Valdés *et al.*, 2006; present study
Pleurobranchus areolatus (Mörch, 1863)	Caribbean Sea, Brazil	Northeastern (Fernando de Noronha, Alagoas*); Southeastern (Rio de Janeiro)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Pleurobranchus atlanticus (Abbot, 1949)	Bermuda, Bahamas, Caribbean Sea, Brazil	Northern (Maranhão); Northeastern (Alagoas*)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
DORIDOIDEA			
Cadlina rumia (Marcus, 1955)	Florida to Brazil; Ghana	Northeastern (Alagoas*, Bahia); Southeastern (Rio de Janeiro, São Paulo)	Edmunds, 1981; Valdés *et al.*, 2006; García García *et al.*, 2008; Ourives *et al.*, 2011; present study
Chromodoris binza (Marcus & Marcus, 1963)	Florida, Caribbean Sea, Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro, São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Chromodoris paulomarcioi (Domínguez, García & Troncoso, 2006)	Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro); southern (Santa Catarina)	García García *et al.*, 2008; Padula *et al.*, 2011; present study
Dendrodoris cf. *krebsii* (Mörch, 1863)	Bahamas, Florida to Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro, São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study

Continued

Table 2. Continued

Taxon	Geographical distribution	Distribution in Brazil	Source
Diaulula greeleyi (MacFarland, 1909)	Eastern Pacific; Bahamas, Florida, Brazil	Northeastern (Alagoas); Southeastern (Rio de Janeiro, São Paulo)	MacFarland, 1909; Rios, 1994; Valdés *et al.*, 2006; present study
Discodoris branneri (MacFarland, 1909)	Bahamas, Florida to Brazil	Northeastern (Pernambuco, Alagoas); Southeastern (Rio de Janeiro, São Paulo)	MacFarland, 1909; García García *et al.*, 2008; Dayrat, 2010; present study
Discodoris voniheringi (MacFarland, 1909)	Brazil	Alagoas	MacFarland, 1909; Dayrat, 2010
Geitodoris pusae (Er. Marcus, 1955)	Costa Rica, Jamaica, Martinique, Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro, São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Hoplodoris hansrosarum (Domínguez, García & Troncoso, 2006)	Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro)	García García *et al.*, 2008; present study
Platydoris angustipes (Mörch, 1863)	Florida, Caribbean Sea, Brazil	Northeastern (Fernando de Noronha, Alagoas*, Bahia)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Rostanga byga (Er. Marcus, 1958)	Bermuda, Bahamas, St Lucia, Mexico, Brazil, Argentina	Northeastern (Ceará, Alagoas*); Southeastern (São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Taringa telopia (Marcus, 1955)	Caribbean Sea, Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro, São Paulo)	Valdés *et al.*, 2006; García García *et al.*, 2008. present study
Taringa sp. 1	Brazil	Alagoas*	Present study
Taringa sp. 2	Brazil	Alagoas*	Present study
AEOLIDIOIDEA			
Berghia rissodominguezi (Muniain & Ortea, 1999)	Florida, Caribbean Sea, Brazil, Argentina	Northeastern (Alagoas*); Southeastern (São Paulo), Southern (Santa Catarina)	Padula *et al.*, 2011; present study
Eubranchus sp.	Brazil	Alagoas	Present study
Flabellina dushia (Marcus & Marcus, 1963)	Bahamas, Florida, Caribbean Sea, Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Flabellina engeli (Marcus & Marcus, 1968)	Florida, Caribbean Sea, Brazil	Northeastern (Alagoas*); Southeastern (Rio de Janeiro, São Paulo), Southern (Santa Catarina)	Valdés *et al.*, 2006; DaCosta *et al.*, 2007; García García *et al.*, 2008; present study
Glaucus atlanticus (Forster, 1777)	Cosmopolitan	Northeastern (Alagoas*, Bahia), Southeastern (São Paulo), Southern (R.G. do Sul)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Nanuca sebastiana (Marcus, 1957)	Caribbean Sea, Brazil	Northeastern (Alagoas*, Pernambuco); Southeastern (Rio de Janeiro)	Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Phidiana lynceus (Bergh, 1867)	Florida, Caribbean Sea, Brazil; Canary Islands and Ghana	Northeastern (Alagoas*, Bahia); Southeastern (Rio de Janeiro, São Paulo)	Edmunds, 1975; Cervera *et al.*, 2004; Valdés *et al.*, 2006; García García *et al.*, 2008; present study
Spurilla neapolitana (delle Chiaje, 1844)	Circumtropical and temperate seas	Northeastern (Pernambuco, Alagoas, Bahia); Southeastern (Rio de Janeiro, São Paulo)	MacFarland, 1909; Valdés *et al.*, 2006; García García *et al.*, 2008; present study

*, new records from Alagoas (present study).

Northeastern Brazilian coast, for most of the species, fill a gap in their distribution records between Southeastern Brazil and the Caribbean Sea (Valdés *et al.*, 2006). This is the case for *Elysia subornata*, *Chromodoris binza* and *Flabellina engeli*, among others (Table 2).

In addition to their importance as additions to knowledge about Alagoas reef biodiversity, and also because some reefs have been impacted by human activities (Correia & Sovierzoski, 2009), the new records provide important data for future investigations such as biogeographical studies. The recent biogeographical hypothesis proposed for Brazilian opisthobranchs is not conclusive, because of the sparse data available from most of the Brazilian coast (García García *et al.*, 2008). For example, the biogeographical region that includes Alagoas plus the coasts of two other Brazilian states ('EBS') was represented by only 30 species (García García *et al.*, 2008: 198). The present study nearly doubled the number of species known from this region. This emphasizes that only through further surveys and taxonomic studies, resulting in a comprehensive list of species from most areas of the Brazilian coast, can a comprehensive biogeographical study be conducted and an informative overview be obtained. In addition, new detailed morphological studies and the use of molecular tools are recommended to identify possible cryptic opisthobranch species in the tropical western Atlantic, as recently reported for sponges (Valderrama *et al.*, 2009), polychaetes (Barroso *et al.*, 2010) and reef fishes (Luiz Jr *et al.*, 2009; Bernal & Rocha, 2011).

ACKNOWLEDGEMENTS

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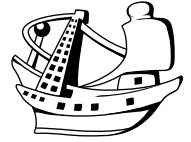V. Padula
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3. RESULTS

Chapter 3

Camacho-García Y, Pola M, Carmona L, **Padula V**, Villani G, Cervera L (2014) Diversity and distribution of the heterobranch sea slug fauna on the Caribbean of Costa Rica. *Cahiers de Biologie Marine* **55**: 109-127.

Diversity and distribution of the heterobranch sea slug fauna on the Caribbean of Costa Rica

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Abstract: The Caribbean is one of the richest areas for heterobranch sea slugs (traditionally called 'opisthobranchs') in the Atlantic. However, detailed information is lacking for most species. Recent studies have highlighted the importance of obtaining more data on Caribbean species. The present study documents new records of opisthobranchs resulting from fieldwork along the Caribbean coast of Costa Rica. A total of 70 species were collected and identified, 17 of which represent new records to Costa Rica. This increases the total known opisthobranch diversity to 152 species in the studied area. Potential species complexes were identified and commented. Future surveys, detailed species reports and comparative morphological and molecular studies are needed to improve the knowledge on the Caribbean opisthobranch fauna.

Résumé : *Diversité et distribution des limaces de mer hétérobranches des côtes caraïbes du Costa-Rica.* Les Caraïbes sont une des régions les plus riches pour les "limaces de mer" hétérobranches (appelées traditionnellement "opisthobranches" en Atlantique). Toutefois les informations manquent sur beaucoup d'espèces. Des études récentes ont mis en évidence l'importance d'obtenir plus d'informations sur les espèces caribbéennes pour mieux comprendre l'importance de la biodiversité des opisthobranches dans cette région. La présente étude présente de nouvelles descriptions d'opisthobranches résultant de travaux d'observation le long des côtes caraïbes du Costa-Rica. Au total, 70 espèces ont été collectées et identifiées, 17 d'entre elles sont observées pour la 1 ère fois au Costa-Rica. Ceci porte le total de la diversité des opisthobranches à 152 espèces dans la zone étudiée. Des espèces complexes potentielles ont été identifiées et commentées. Dans l'avenir, une description détaillée des espèces ainsi que des études de la morphologie comparée et des études moléculaires seront nécessaires pour améliorer les connaissances sur la faune d'opisthobranches dans toute la zone des Caraïbes.

Introduction

The Caribbean is one of the richest areas for opisthobranchs in the Atlantic (Valdés et al., 2006; García & Bertsch, 2009). Herein, we use the traditional name "opisthobranchs," but as an informal group, since the rejection of the monophyly of the Opisthobranchia has been confirmed by recent studies (Klussmann-Kolb et al., 2008; Jörger et al., 2010; Dinapoli & Klussmann-Kolb, 2010; Dinapoli et al., 2011; Schrödl et al., 2011). In the past 60 years, several papers have been published on opisthobranchs from the Caribbean, including descriptions of at least 120 new species to science, with the greatest contribution of all by the researchers Ernst & Eveline Marcus (e.g. 1967 & 1970). However, the quantity of publications is deceptive in regards to what is truly known about the fauna of this region. Most works were based on reduced number of specimens, lacking photos and detailed descriptions, as also precise information on collection localities and species habitat. This has not only left many gaps in the knowledge of the fauna of this region, but has also raised doubts on the validity of some species. As a result, much work remains to be done throughout the Caribbean and, as commented by Valdés et al. (2006: 8), "some of the (Caribbean) species are in need to study using modern techniques and consequently some of the identifications are provisional" and "we expect to have to make changes and revisions in the near future."

The first records of opisthobranchs along the Caribbean coast of Costa Rica were provided among general lists of marine mollusks found in this area (Houbrick, 1968; Robinson & Montoya, 1987). Several studies from 1995 to 2004 focused on the opisthobranch fauna from this region, resulting in a list of 91 species, including new records (e.g., Espinosa & Ortea, 2001). As a result of these studies many new species were also described (Caballer et al., 2001; Espinosa & Ortea, 2001; Ortea, 2001; Ortea & Espinosa, 2000 & 2002; Ortea et al., 2001, 2003 & 2004). More recently, Valdés et al. (2006) reported a total of 105 species from the Caribbean of Costa Rica, including 11 new records. The same authors listed 12 species recently described or found in Costa Rica as potential synonyms with species previously known from the tropical Western Atlantic. For example, *Elysia eugeniae* Ortea & Espinosa, 2002 as synonym of *Elysia canguzua* Er. Marcus, 1955; *Polycera manzanilloensis* Ortea, Espinosa & Camacho, 1999 as synomym of *P. herthae* Ev. Marcus & Er. Marcus, 1963; and *Millereolidia ritmica* (Ortea, Caballer & Espinosa, 2003), as synonym of *Berghia creutzbergi* Er. Marcus & Er. Marcus, 1970. Lastly, Camacho-García (2009) documented a total of 123 species of opisthobranchs from along the Caribbean coast of Costa Rica, compiled

Figure 1. Collecting sites along the Caribbean coast of Costa Rica. **A.** Cahuita National Park. **B.** Collecting localities at Punta Uva and Gandoca-Manzanillo National Wildlife Refuge. Scale bar: 1 km.

Table 1. Collecting localities along the Caribbean coast of Costa Rica.

Location name	Station code	Coordinates
(1) Cahuita National Park (CNP):	CNP1	09º43.64'N-082º48.63'W
	CNP2	09º44.25'N-082º 48.62'W
Punta Vargas	PV3	09º44.41'N-082º48.29'W
Punta Vargas	PV4	No coordinates available
	CNP5	09º44.39'N-082º48.49'W
	CNP6	09º45.04'N-082º49.17'W
	CNP7	09º44.45'N-082º48.21'W
	CNP8	09º44.60'N-082º48.30'W
	CNP9	09º44.22'N-082º47.94'W
Jardín Eduardo	JE10	No coordinates available
Punta Cahuita	PC11	09°45.05'N-082º48.54'W
(2) Gandoca-Manzanillo National Wildlife Refuge (GMRNVS):		
Punta Mona	PM12	09º37.84'N-082º37.06'W
Punta Uva, in front of Aguas Claras Hotel	ACH13	09º38.39'N-082º43.06'W
Playa de Piedras Blancas	PPB14	09º38.13'N-082º38.42'W
Off shore Manzanillo	OSM15	09º39.46'N-082º39.75'W
Jardín del coral	JC16	No coordinates available
Manzanillo reef	MR17	09º38.10'N-082º39.27'W
(3) Punta Uva	PU18	09º38.48'N-082º41.61'W

information about species biogeography and the country's voucher collections, and provided recommendations for future studies in the area. The present study updates the knowledge on the opisthobranch fauna of the Caribbean coast of Costa Rica. New distributional range and morphological data are presented, including color photos of uncommon and potential complexes of species.

Material and Methods

Sampling was carried out between 11-20 May 2011 along the Caribbean coast of Costa Rica.

All specimens were collected with a permit from the Sistema Nacional de Áreas de Conservación (181-2010-SINAC), and deposited at Museo de Zoología de la Universidad de Costa Rica (MZUCR). The specimens were collected in intertidal and subtidal areas during the day at 18 different locations (Table 1 & Fig. 1). The collecting sites were surveyed through snorkeling or SCUBA diving up to 25 meters deep. The specimens were obtained using direct (collecting observed animals) and indirect methods (collecting algae and hydrozoans for later observation). Specimens were photographed and measured alive, then relaxed in a solution of $MgCl_2$ with saltwater. After relaxation, the specimens were preserved in 95% ethyl alcohol. The taxonomic identification was based on external color and morphology, in comparison to the Caribbean Sea Slugs field guide (Valdés et al., 2006) and original descriptions (MacFarland, 1909; Er. Marcus, 1955; Ev. Marcus,

1971; Ev. Marcus & Er. Marcus, 1967; Ortea & Espinosa, 2002; Ortea et al., 2001; among others). In some cases, the internal anatomy was studied to confirm species identifications. The synonymy lists presented here are not exhaustive, and in some cases refer to published works where a complete list can be found. Taxonomic nomenclature was considered on a species by species basis since recently changes were proposed for some groups, such as Chromodorididae (Johnson & Gosliner, 2012) and Discodorididae (Dayrat, 2010). Comments are included in every case when we disagree with the recently proposed nomenclature.

Results

A total of 70 species were collected, increasing the total known opisthobranch diversity to 152 species in the Caribbean of Costa Rica. In total, 17 species are new records for Costa Rica: *Haminoea* cf. *antillarum* (d'Orbigny, 1841), *Ascobulla ulla* (Er. Marcus & Ev. Marcus, 1970), *Elysia subornata* Verril, 1901, *Elysia* sp., *Thuridilla* sp., *Cyerce* cf. *cristallina* (Trinchese, 1881), *Berthella* sp., *Berthellina quadridens* Mörch, 1863, *Diaulula hummelincki* (Ev. Marcus & Er. Marcus, 1963), *Sclerodoris worki* (Ev. Marcus & Er. Marcus, 1967), Discodorididae sp. 1, *Doriopsilla espinosai* Valdés & Ortea, 1998, *Doriopsilla pharpa* Er. Marcus, 1961, *Tritonia* cf. *pickensi* Ev. Marcus & Er. Marcus, 1967, *Limenandra nodosa* Haefelfinger & Stamm, 1958, *Flabellina dushia*

(Ev. Marcus & Er. Marcus, 1963) and *Learchis evelinae* Edmunds & Just, 1983. For most of these species, the records from the coast of Costa Rica represent important range extensions on their geographic distribution as they had never been previously collected west of Jamaica, Cuba, or Colombia (e.g., *Cyerce* cf. *cristallina*, *Doriopsilla espinosai,* and *Thuridilla* sp., respectively), whereas a few others have been reported for the western Caribbean (e.g. Belize, Honduras) but not from Costa Rica. Of the 18 collecting sites, Punta Mona R.N.V.S. Gandoca-Manzanillo, Limón (09º37.849' N-82º37.065' W), was the locality with the highest number of species (35).

Systematics

CEPHALASPIDEA Fischer, 1887
Family Aglajidae Pilsbry, 1895-96
Navanax gemmatus (Mörch, 1863)

Synonyms

For a complete list of synonyms see Ornelas-Gatdula et al. (2012).

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8310, MZUCR8314), L: 10-20 mm respectively. Subtidal 6.8 m deep, under rocks.

Remarks

A recent study conducted by Ornelas-Gatdula et al. (2012) concluded that *Navanax aenigmaticus* comprises a complex of three cryptic species with disjunct ranges in the eastern Pacific, western Atlantic, and eastern Atlantic. *Navanax gemmatus* (Mörch, 1863) is the valid name for the western Atlantic species.

Family Haminoeidae Pilsbry, 1893
Haminoea cf. *antillarum* (d'Orbigny, 1841)
(Fig. 2A)

Synonyms

See Valdés et al. (2006) for a complete list of synonyms.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8397), L: 10 mm. Subtidal 4 m deep, under rocks.

Morphological comments

See Valdés et al. (2006) for a complete description. The specimen collected here has a background color with high

concentrations of black pigment and a few areas of translucent gray. There are a few opaque white and orange dots all over the body that are more obvious when present inside the translucent gray area. Several orange, black, and a few white dots are visible through the shell.

Remarks

Due to the recent findings of cryptic species in the genus *Haminoea* (Malaquias, unpubl. data) a more thorough morphological and molecular study is necessary to truly clarify the identity of this species. This taxon is a new record for Costa Rica.

Haminoea elegans (Gray, 1825)

Synonyms

Bulla elegans Gray, 1825: 408, *Bulla diaphana* Gould, 1852: 222; *Bulla guildingii* Swainson, 1840: 360, fig. 46; *Haminoea taylorae* Petuch, 1987: 31-32, pl. 4, figs. 12-13.

Material

Cahuita National Park (CNP9): 5 specimens (MZUCR8282, MZUCR8283, MZUCR8284, MZUCR8285, MZUCR8286), L: each 7 mm. Subtidal 4 m deep, sand bottoms.

SACOGLOSSA Von Ihering, 1876
Family Volvatellidae Pilsbry, 1895
Ascobulla ulla (Er. Marcus & Ev. Marcus, 1970)
(Fig. 2B)

Synonym

Cylindrobulla ulla Er. Marcus & Ev. Marcus, 1970: 25-26, fig. 33.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8417), L: 9 mm, including shell and soft part. Subtidal 4 m deep, on *Caulerpa* sp.

Remarks

This species is a new record for Costa Rica.

Family Juliidae E. A. Smith, 1885
Berthelinia caribbea Edmunds, 1963

Material

Cahuita National Park (CNP9): 1 specimen (MZUCR8290); RNVS, Gandoca-Manzanillo (PB14): 1 specimen (MZUCR8372), L: 4 mm each. Subtidal 4 m deep, on *Halimeda* sp.
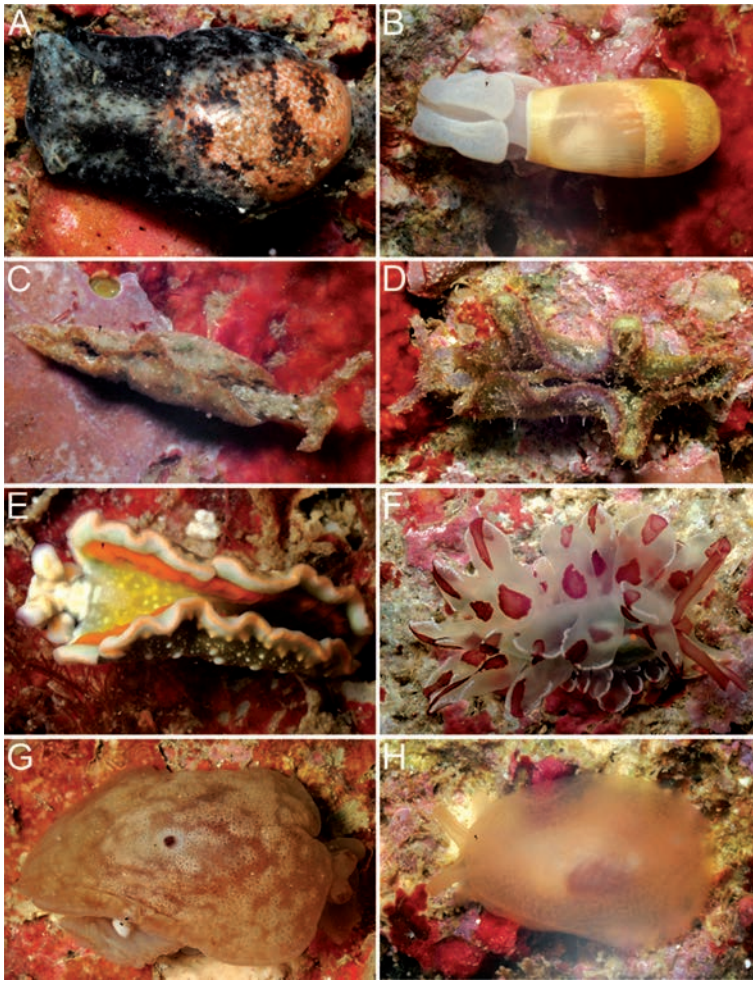
Figure 2. New opisthobranch records from the Caribbean coast of Costa Rica. **A.** *Haminoea* cf. *antillarum* (MZUCR8397, L: 10 mm). **B.** *Ascobulla ulla* (MZUCR8417, L: 9 mm including shell and body). **C.** *Elysia subornata* (MZUCR8464, L: 6 mm). **D.** *Elysia* sp. (MZUCR8376, L: 15 mm). **E.** *Thuridilla* sp. (MZUCR8236, L: 10 mm). **F.** *Cyerce* cf. *cristallina* (MZUCR8299, L: 15 mm). **G.** *Berthella* sp. (MZUCR8203, L: 25 mm). **H.** *Berthellina quadridens* (MZUCR8436, L: 8 mm).

Family Oxynoidae Stoliczka, 1868 (1847)
***Lobiger souverbii* P. Fischer, 1857**

Synonym

Lobiger pilsbryi Schwengel, 1941: 37-40, pl. 3, figs. 1-5

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR 8416), L: 9 mm. Subtidal 4 m deep, on *Caulerpa* sp.

***Oxynoe antillarum* Mörch, 1863**

Synonym

?*Oxynoe aguayoi* Jaume, 1945: 22-23, pl. 2, fig. 6

Material

Cahuita National Park (CNP2): 2 specimens (MZUCR8207, MZUCR8208), L: 5-25 mm. Subtidal 3-4 m deep, on *Caulerpa* sp. and *Sargassum* sp.

Family Plakobranchidae Gray, 1840
***Elysia canguzua* Er. Marcus, 1955**

Synonym

Elysia eugeniae Ortea & Espinosa, 2002: 130-133, figs. 1A-C, 2A-D; pl. 1, fig. A.

Material

Cahuita National Park, Limón (CNP7): 4 specimens (MZUCR8267, MZUCR8268, MZUCR8269, MZUCR8270), L: 4-7 mm. Subtidal 4 m deep, on *Codium* sp.

***Elysia crispata* Mörch, 1863**

Synonyms

Tridachia schrammi Mörch, 1863: 41, *Elysia verrilli* Pruvot-Fol, 1946: 39 [non *Elysia verrilli* Thiele, 1931], *Elysia* (*Elysiopterus*) *pruvotfolae* Er. Marcus, 1957: 415, *Tridachia whiteae* Er. Marcus, 1957: 416

Material

Cahuita National Park (CNP1): 9 specimens (MZUCR8199, MZUCR8200, MZUCR8201, MZUCR8202, MZUCR8231, MZUCR8232, MZUCR8246, MZUCR8315, MZUCR8316), L: 25-40 mm. Subtidal 2-25 m deep, on rocks.

***Elysia flava* Verril, 1901**

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8333, MZUCR8410), L: 4-7 mm. Subtidal 4-6 m deep, under rocks.

***Elysia ornata* (Swainson, 1840)**

Synonyms

Thallepus ornatus Swainson, 1840: 251, 359; *Pterogasteron marginatum* Pease, 1871: 304, pl. 21, fig. 3.

Material

Cahuita National Park (CNP1): 9 specimens (MZUCR8190, MZUCR8191, MZUCR8192, MZUCR8193, MZUCR8194,

MZUCR8221, MZUCR8222, MZUCR8223, MZUCR 8245), L: 1-15 mm; RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8402), L: 20 mm. Subtidal 3-13 m deep, on green algae.

Elysia patina Ev. Marcus, 1980
(Fig. 4A)

Material

Cahuita National Park (CNP7): 1 specimen (MZUCR8276), L: 5 mm. RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8465), L: 5 mm. Subtidal 4-5 m deep, on *Codium* sp. and on *Avrainvillea longicaulis*.

Elysia subornata Verril, 1901
(Fig. 2C)

Synonym

Elysia cauze Er. Marcus, 1957: 405.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8376), L: 15 mm. RNVS, Gandoca-Manzanillo (MR17): 3 specimens (MZUCR8462, MZUCR8463, MZUCR8464), L: 4-6 mm. Subtidal 5-6 m deep, under rocks or on *Avrainvillea longicaulis*.

Remarks

This species is a new record for Costa Rica.

Elysia tuca Ev. Marcus & Er. Marcus, 1967

Material

Cahuita National Park (CNP2): 8 specimens (MZUCR8217, MZUCR8224, MZUCR8261 to MZUCR8266). RNVS, Gandoca-Manzanillo (PPB14): 2 (MZUCR8368, MZUCR8369) and 7 specimens (MZUCR8373), L: 3-13 mm. Subtidal 2-4 m deep, on *Halimeda* sp. and *Codium* sp.

Elysia zuleicae Ortea & Espinosa, 2002
(Fig. 4B)

Material

RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8466), L: 8 mm. Subtidal 5 m deep, on *Avrainvillea longicaulis.*

Elysia sp.
(Fig. 2D)

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8376), L: 15 mm. Subtidal 5 m deep, under a rock.

Morphological comments

Dark green background with light brown irregular patches and light blue areas near the mouth, behind the head, and near the borders of the slight undulated parapodia.

Remarks

This specimen belongs to an undescribed species, which resembles *Elysia subornata* and *Elysia papillosa*. Previously known from Florida (K. Jensen pers. comm.), herein for the first time reported from Costa Rica.

Thuridilla mazda Ortea & Espinosa, 2000

Material

Cahuita National Park (PV3): 1 specimen (MZUCR8218), L: 2 mm. Subtidal 5.4 m deep, on a rock.

Thuridilla picta (Verril, 1901)

Synonyms

Elysia picta Verrill, 1901: 30-31, pl. 4, fig. 2, *Elysia duis* Ev. Marcus & Er. Marcus, 1967a: 31-32, figs. 33-37.

Material

RNVS, Gandoca-Manzanillo (JC16): 1 specimen (MZUCR8437), L: 10 mm. Subtidal 25 m deep, on a rock.

Thuridilla sp.
(Fig. 2E)

Material

Cahuita National Park (CNP5): 3 specimens (MZUCR8235, MZUCR8236, MZUCR8237), L: 10-12 mm. Subtidal 3 m deep, on rocks.

Morphological comments

For morphological description, see Valdés et al. (2006: 60-61). In addition, there are two orange bands in the dorsal area that are clearly visible when the parapodia are not folded over to enclose the body.

Remarks

There are three known Atlantic species of *Thuridilla*: *T. hopei* (occurring in the Mediterranean and E. Atlantic, including Azores, Madeira and Canary Archipelagos; Carmona et al., 2011, Malaquias et al., 2012), *T. picta* (Caribbean Sea, including Bermuda, Carmona et al., *op. cit.*, Malaquias et al., *op. cit.*), and *T. mazda* (Caribbean Sea and Azores archipelago; Malaquias et al., *op. cit.*), all of which are clearly different from this form. This undescribed species was previously recorded from Florida, Granada and Colombia (Valdés et al., 2006).
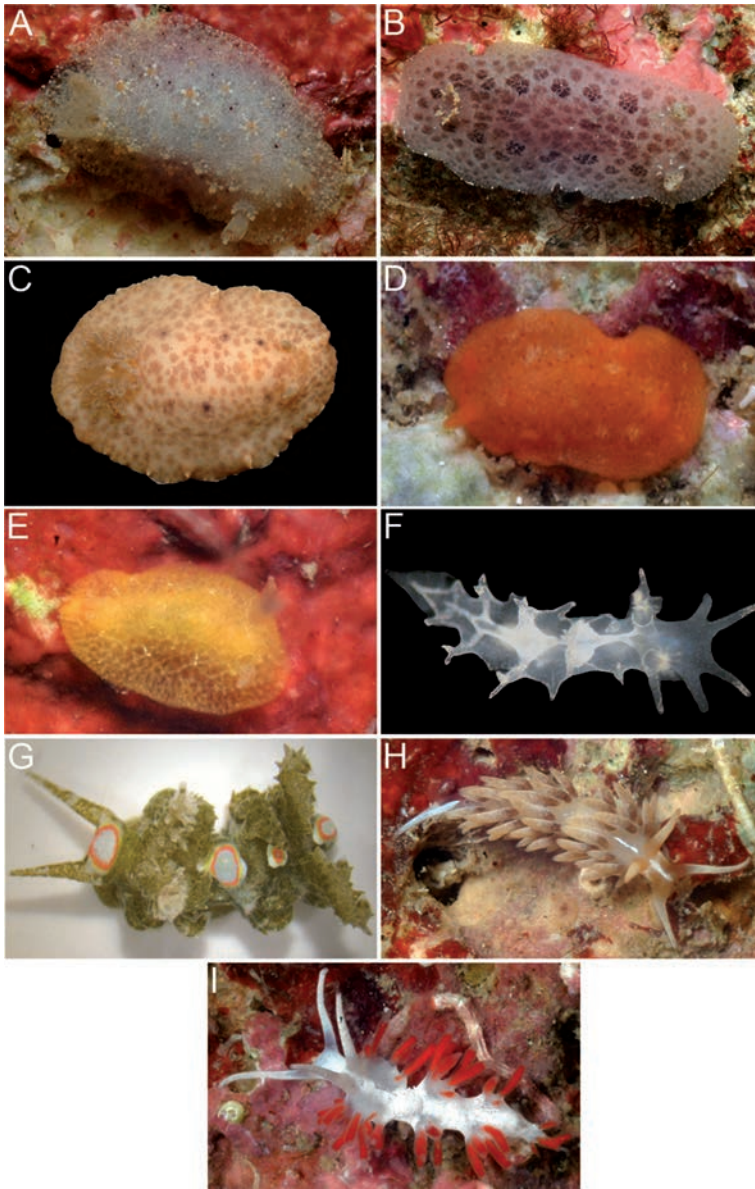
Figure 3. New opisthobranch records from the Caribbean coast of Costa Rica. **A.** *Sclerodoris worki* (MZUCR8401, L: 14 mm). **B.** *Diaulula hummelincki* MZUCR8343, L: 8 mm). **C.** Discodorididae sp.1 (MZUCR8259, L: 70 mm). **D.** *Doriopsilla espinosai* (MZUCR8291, L: 3.5 mm). **E.** *Doriopsilla pharpa* (MZUCR8244, L: 3 mm). **F.** *Tritonia* cf. *pickensi* (MZUCR8300, L: 2 mm). **G.** *Limenandra nodosa* (MZUCR8344, L: 4 mm). **H.** *Learchis evelinae* (MZUCR8277, L: 5 mm). **I.** *Flabellina dushia* (MZUCR8252, L: 4 mm).

Family Hermaeidae H. Adams & A. Adams, 1854
Hermaea cf. coirala Er. Marcus, 1955
(Fig. 4C)

Material

RNVS, Gandoca-Manzanillo (PPB14): 1 specimen (MZUCR8371), L: 3 mm. Subtidal 3.5 m deep.

Morphological comments

Background color transparent, with several opaque white spots. The brown digestive system can be seen through the body. There are seven large translucent cerata, and a couple of smaller ones, with numerous opaque white spots mostly in the apical zone. The brown digestive branches with distal cruciform shape can also be seen through the cerata.

Remarks

There are three species of *Hermaea* described from the Atlantic: *Hermaea bifida* (Montagu, 1815) originally described from southwestern United Kingdom, *Hermaea coirala* (Er. Marcus, 1955) from southeastern Brazil, and *Hermaea cruciata* Gould, 1870 which occurs from Massachusetts to east Florida. Some authors and databases report *H. coirala* as a synonym of *H. cruciata* (see Valdés et al., 2006; Rosenberg, 2009), mainly due to the similarity in the digestive branching shape. In fact, it is not clear how much variable this character is, and therefore the group needs a taxonomic revision. Due to the small length of our specimen and the fact that *H. cruciata* was described from a temperate region, we prefer to identify our material as *H.* cf. *coirala*.

Family Limapontiidae Gray, 1847
***Costasiella nonatoi* (Ev. Marcus & Er. Marcus, 1960)**

Material

RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8458), L: 1 mm. Subtidal 5 m deep, on *Avrainvillea longicaulis*.

***Costasiella ocellifera* (Simroth, 1895)**

Synonyms

Doto ocellifera Simroth, 1895: 168-170, pl. 20, figs. 6-10, *Stiliger lilianae* Ev. Marcus & Er. Marcus, 1969: 7-12, figs. 22-28.

Material

RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8459), L: 0.5 mm. Subtidal 5 m deep, on *Avrainvillea longicaulis*.
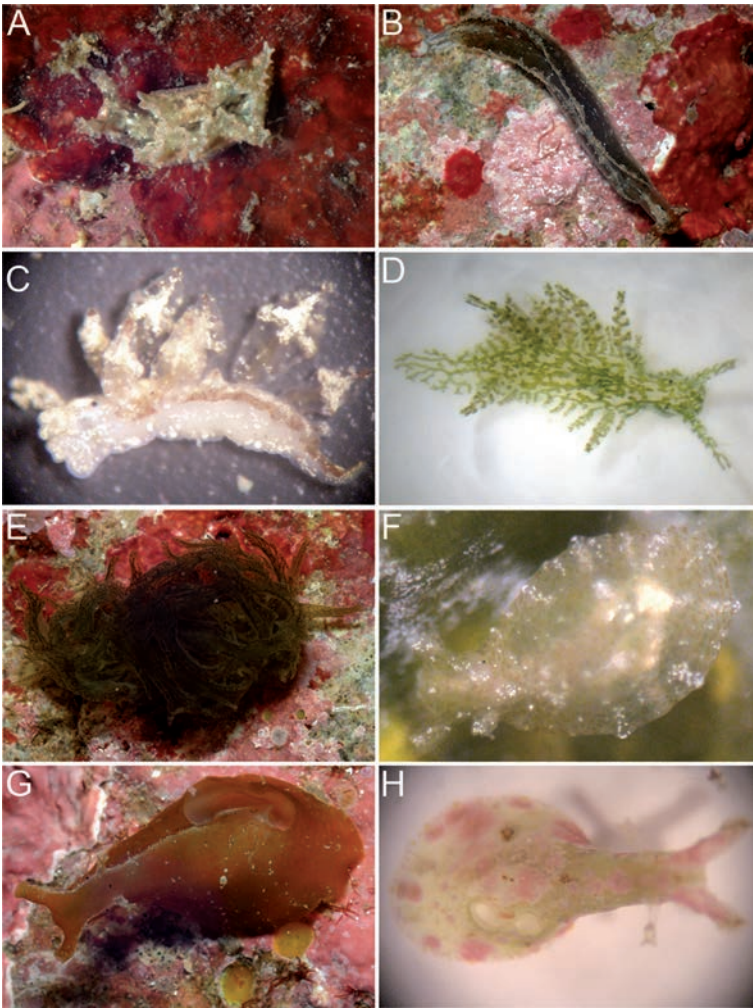
Figure 4. Uncommon species found along the Caribbean coast of Costa Rica. **A.** *Elysia patina* (MZUCR8276, L: 5 mm). **B.** *Elysia zuleicae* (MZUCR8466, L: 8 mm). **C.** *Hermaea* cf. *coirala* (MZUCR8371, L: 3 mm). **D.** *Placida verticillata* (MZUCR8275, L: 4 mm). **E.** *Caliphylla* sp. (MZUCR8406, L: 20 mm). **F.** *Notarchus punctatus* (MZUCR8243, L: 3 mm). **G.** *Petalifera* sp. (MZUCR 8345, L: 8 mm. **H.** *Phyllaplysia* sp (MZUCR8444, L: 5 mm).

Placida verticillata Ortea, 1981
(Fig. 4D)

Material

RNVS, Gandoca-Manzanillo (PM12): 10 specimens (MZUCR8271 to MZUCR8275, MZUCR8279, MZUCR 8280, MZUCR8281, MZUCR8412, MZUCR8415), L: 3-4 mm. Subtidal 3-5 m deep, on *Codium* sp.

Remarks

No external morphological differences have been found between our specimen and the data available on specimens from the eastern Atlantic, where the species was originally described. No data exist on the type-development of this

species, although other congeneric species seem to have planktotrophic development (Jensen, 2001). A study to test the conspecific status of populations on both sides of the Atlantic Ocean is necessary.

Family Caliphyllidae Tiberi, 1881
Caliphylla **sp.**
(Fig. 4E)

Material

RNVS, Gandoca-Manzanillo (PM12): 4 specimens (MZUCR8386, MZUCR8404, MZUCR8405, MZUCR8406), L: 7-20 mm. Subtidal 5.5 m deep, on *Bryopsis* sp.

Remarks

As Valdés et al. (2006) commented, one of the main differences between this species and *Caliphylla mediterranea* are the elongate cerata in *Caliphylla* sp. This is probably an undescribed species only known from Costa Rica and Tobago (Valdés et al., 2006).

Cyerce antillensis **Engel, 1927**

Synonym

Cyerce habanensis Ortea & Templado, 1988: 11-14, figs. 1-2.

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8338, MZUCR8408), L: 9-15 mm; Punta Uva (PU18): 1 specimen (MZUCR8423), L: 17 mm; Cahuita National Park (CNP1): 1 specimen (MZUCR8225), L: 3 mm. Subtidal 3-7 m deep, under rocks.

Cyerce **cf.** *cristallina* **(Trinchese, 1881)**
(Fig. 2F)

Synonyms

Lobiancoia cristallina Trinchese, 1881: 116, *Cyerce iheringi* Pelsenner, 1892: 19.

Material

Cahuita National Park (CNP10): 1 specimen (MZUCR 8299), L: 15 mm. Subtidal 3.3 m deep, under a rock.

Morphological comments

For a complete description, see Valdés et al. (2006). Our single specimen matches the description of the specimens

from Valdés et al. (2006) identified as *Cyerce cristallina*, with the difference that the red color is darker on the head, rhinophores, and the cerata in our specimen.

Remarks

Although Valdés et al. (2006) report records of this species from several Caribbean regions, this is the first time it has been recorded from Costa Rica. However, some clarifications should be made about the specific identity of the western Atlantic material attributed to *C. cristallina*. This species was described by Trinchese (1881) based on material collected from the Gulf of Naples (Mediterranean Sea), and the Mediterranean records are only from middle and eastern Mediterranean (see Schmekel & Portmann, 1982; Thompson, 1988), but not from the Iberian Peninsula nor from the Macaronesian archipelagos. Other known records are from Caribbean regions and Bermuda. Jensen (2001) reported data on the development of *C. cristallina* from the Gulf of Naples supplied by Schmekel & Portmann (1982), tentatively considering it planktotrophic (free-swimming veligers hatch after 22 days at 16ºC). No data exist on the type-development of individuals from the western Atlantic. Moreover, Thompson (1988) indicated the presence of a spine at the tip of the penis of the Jamaican individuals that has not been verified in the Mediterranean material, as well as some minor color differences between material from the Mediterranean and Caribbean regions. For these reasons, the conspecific status of both populations should not be assumed until a study is carried out. A molecular analysis of the amphiatlantic status of some sacoglossans has been published recently (Carmona et al., 2011), assuming this status for some species but rejecting it for others. This species is recorded for the first time for Costa Rica, and it has not been reported from other localities west of Jamaica (Valdés et al., 2006).

Mourgona germaineae Er. Marcus & Ev. Marcus, 1970

Material

Punta Uva (PU18): 1 specimen (MZUCR8439), L: 13 mm. Subtidal 3 m deep, on *Caulerpa*. sp.

APLYSIOMORPHA Pelseneer, 1906
Family Aplysiidae Lamarck, 1809
Aplysia dactylomela Rang, 1828

Synonyms

For a complete list of synonyms see Martínez (1995).

Material

Punta Uva (ACH13): 2 specimens (MZUCR8309, MZUCR8346), L: 40-45 mm; RNVS, Gandoca-Manzanillo

(MR17): 2 specimens (MZUCR8452, MZUCR8461), L: 11-33 mm. Subtidal 5-7 m deep, under rocks.

Aplysia parvula Mörch, 1863

Synonyms

For a complete list of synonyms see Martínez (1995).

Material

Cahuita National Park (CNP1): 4 specimens (MZUCR8195, MZUCR8196, MZUCR8197, MZUCR8198), L: 10-15 mm; Punta Uva (PU18): 1 specimen (MZUCR8428), L: 15 mm. Subtidal 3-13 m deep, in areas of seagrass.

Dolabrifera dolabrifera (Cuvier, 1817)

Synonyms

For a complete list of synonyms see Martínez (1995).

Material

RNVS, Gandoca-Manzanillo (PM12): 3 specimes (MZUCR8307, MZUCR8308, MZUCR8347), L: 10-30 mm; RNVS, Gandoca-Manzanillo (PPB14): 1 specimen (MZUCR8356), L: 40 mm; Off shore Manzanillo (OSM15): 1 specimen (MZUCR8370), Punta Uva (PU18): 2 specimens (MZUCR8429, MZUCR8430), L: 39-50 mm; RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8472), L: 15 mm. Subtidal 3-11 m deep, under rocks and on grassy areas.

Notarchus punctatus Philippi, 1836
(Fig. 4F)

Synonyms

Notarchus punctatus Philippi, 1836; *Notarchus neapolitanus* delle Chiaje, 1841.

Material

Cahuita National Park (CNP1): 1 specimen (MZUCR8243), L: 3 mm. Subtidal 8 m deep, on hydrozoans.

Petalifera sp.
(Fig. 4G)

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8345, MZUCR8407), L: 8-13 mm. Subtidal 5.5-7 m deep, found on *Sargassum* or under rocks.

Morphological comments

The background color is light brown with random cream spotting on the dorsum. Small cream ramified papillae are present on the dorsum of the animals. The brownish rhinophores are short, blunt, and auriculate with cream specks. Cephalic tentacles are large and thick with a uniform light brown color. The shell of this animal is quadrangular in the anterior part, with a pronounced anal notch and a conspicuous protoconch, whose apex bends downwards. The shell is well-calcified, translucent white in color, and about 3.5 mm wide and 3.5 mm long.

Remarks

In the Caribbean, only two species of *Petalifera* are known (Valdés et al., 2006): *Petalifera petalifera* (Rang, 1828) and *Petalifera ramosa* Baba, 1959, originally described from the Mediterranean and Japan, respectively (Martínez, 1996). Considering the external morphology only, our specimen differs from *P. petalifera* by the absence of green-brown specks or lines on the dorsum and from *P. ramosa* by absence of conical tubercles scattered over the dorsum. Further morphological and molecular studies should be done in order to clarify the identity of our morphotype.

Phyllaplysia sp.
(Fig. 4H)

Material

Punta Uva (PU18): 1 specimen (MZUCR8444), L: 5 mm. Subtidal 3 m deep, on the alga *Caulerpa* sp.

Morphological comments

Background color translucent greenish, large areas of pink pigment are concentrated on the dorsum and oral tentacles. A few brown spots are also present on the dorsum. Dorsum without tubercles. The specimen lacks a shell.

Remarks

Only two species of *Phyllaplysia* are known from the Caribbean (Williams & Gosliner, 1973; Valdés et al., 2006): *P. engeli* Er. Marcus, 1955, and *P. smaragda* Clark, 1977. These two species lack the large areas of pink pigment that characterize our specimen. Further morphological and molecular studies should be done in order to clarify the identity of our morphotype.

Stylocheilus striatus (Quoy & Gaimard, 1832)

Synonyms

Aplysia striata Quoy & Gaimard, 1832-33 [1832]: 315-316, pl. 24, figs. 9-11; *Notarchus polyomma* Mörch, 1863: 25

Material

RNVS, Gandoca-Manzanillo (PM12): 4 specimens (MZUCR8303, MZUCR8341, MZUCR8394, MZUCR8395), L: 9-28 mm; RNVS, Gandoca-Manzanillo (MR17): 6 specimens (MZUCR8467, MZUCR8468, MZUCR8469, MZUCR8460, MZUCR8470, MZUCR 8471), L: 4-8 mm. Subtidal 4-7 m deep, under or on rocks, and some on brown algae.

NUDIPLEURA Wägele & Willan, 2000
PLEUROBRANCHOMORPHA Pelseneer, 1906
Family Pleurobranchidae Gray, 1827
Berthella sp.
(Fig. 2G)

Material

Cahuita National Park (CNP1): 1 specimen (MZUCR 8203), L: 25 mm. Subtidal 13 m deep, under a rock.

Morphological comments

The background color of the specimen is light brown. The dorsum has several light brown patches and numerous minute brown spots. There is a very conspicuous dark brown spot, located in the center of the dorsum.

Remarks

Only two species of *Berthella* are known from the Caribbean (Valdés et al., 2006): *B. stellata* (Risso, 1826) and *B. agassizii* (MacFarland, 1909). These two species differ from our specimen by the external coloration and the lack of a large dark brown spot at the center of the notum. The same morphotype was reported from Puerto Rico (Valdés et al., 2006) and from the northeastern Brazilian coast (Padula et al., 2012). An externally very similar morphotype was also recorded from different localities in the western and central Pacific (Gosliner et al., 2008). It was pointed out the similarity of the Pacific material to *Berthella africana* (Pruvot-Fol, 1953), originally described from the Morocco. Unfortunatelly, no material was found during recent (2008-2013) fieldwork at several Atlantic Moroccan localities, including the type locality of *B. africana* (Cervera, unpubl. data). The taxonomic status of these specimens needs to be reassessed on the basis of anatomical and molecular studies comparing Pacific and Atlantic material. This morphotype is reported for the first time from Costa Rica.

Berthellina quadridens Mörch, 1863
(Fig. 2H)

Synonyms

Berthella circularis Mörch, 1863: 31, *Pleurobranchus amarillius* Mattox, 1953: 109-114, figs. 1-10.

Material

RNVS, Gandoca-Manzanillo (JC16): 1 specimen (MZUCR8436), L: 8 mm. Subtidal 25 m deep, under a rock.

Remarks

This species is a new record for Costa Rica.

Pleurobranchus crossei Vayssière, 1897

Synonyms

Pleurobranchopsis aurantiaca Verrill, 1900: 547. pl. 66, fig. 5 [non *Pleurobranchus aurantiacus* Risso, 1818], *Pleurobranchus verrilli* Thiele, 1931: 419, *Pleurobranchus atlanticus* Abbott, 1949: 73-77, pl. 5, figs. 1-10D.

Material

Cahuita National Park (CNP9): 2 specimens (MZUCR8295, MZUCR8296), L: 35-55 mm. Subtidal 4 m deep, under a rock.

Pleurobranchus sp.
(Fig. 5A)

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8337), L: 5 mm; RNVS, Gandoca-Manzanillo (MR17): 2 specimens (MZUCR8453, MZUCR8454), L: 3-5 mm. Subtidal 7 m deep, under a rock.

Morphological comments

The background color is translucent white to reddish with numerous opaque white spots all over the body, the rhinophores, and oral tentacles. The mantle margin, rhinophores, and oral tentacles have an orange line. Some orange tubercles have a reddish line around them.

Remarks

Three species of *Pleurobranchus* are reported from the Caribbean: *Pleurobranchus areolatus* Mörch, 1863; *Pleurobranchus evelinae* Thompson, 1977, and *Pleurobranchus crossei* (Valdés et al., 2006). Although our specimen resembles *P. areolatus* or even *P. crossei*, it is difficult to determine the identity of the specimen since it is a juvenile, and there is a lack of information regarding color forms of the cited species in differents life stages.

NUDIBRANCHIA Blainville, 1814
EUCTENIDIACEA Tardy, 1970
Family Chromodorididae Bergh, 1891
Felimida binza (Ev. Marcus & Er. Marcus, 1963)
(Fig. 5B)

Synomym

Chromodoris binza Ev. Marcus & Er. Marcus, 1963: 25-26, figs. 30-31.

Material

Cahuita National Park (PV3): 1 specimen (MZUCR8220), L: 5 mm.; RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8336), L: 2 mm. Subtidal, 5.5-7 m deep, under rocks.

Remarks

For the Chromodorididae species, we followed the nomenclature recently proposed by Johnson & Gosliner (2012), in which the monophyly of traditionally widespread genera, such as *Chromodoris* and *Hypselodoris*, was not supported. *Felimida binza* is part of a complex whose members are very similar in color and morphology, which includes *Felimida britoi* (Ortea & Pérez, 1983), from the eastern Atlantic and Mediterranean Sea, *Felimida clenchi* (Russel, 1935), and *Felimida neona* (Marcus, 1955) from the tropical Western Atlantic. The boundaries between these species are not clear, due to the occurrence of specimens with intermediate color patterns; the possibility that some names represent synonyms exists. Herein we include specimens with the traditional color form attributed to *F. binza*. The same applies to *F. clenchi* (see below).

Felimida clenchi (Russell, 1935)
(Fig. 5C)

Synonym

Glossodoris clenchi Russell, 1935: 59, fig. 5.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8391), L: 12 mm; Off the shore of Manzanillo (OSM15): 3 specimens (MZUCR8351, MZUCR8352, MZUCR8353), L: 3-10 mm; RNVS, Gandoca-Manzanillo (PPB14): 1 specimen (MZUCR8363) L: 7 mm. Subtidal 3-11 m deep, under rocks.

Remarks

See comments above regarding this species.
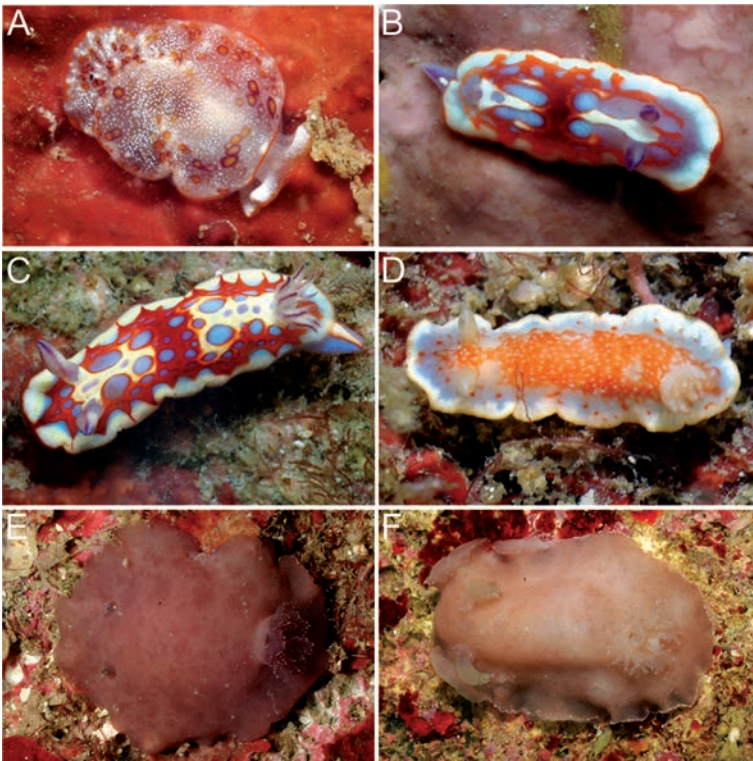
Figure 5. Uncommon species found along the Caribbean coast of Costa Rica. **A.** *Pleurobranchus* sp. (MZUCR8337, L: 5 mm). **B.** *Felimida binza* (MZUCR8220, L: 5 mm). **C.** *Felimida clenchi* (MZUCR8351, L: 10 mm). **D.** *Felimida regalis* (MZUCR8234, L: 6 mm). **E.** *Diaulula phoca* (MZUCR8240, L: 30 mm). **F.** Discodorididae sp. 2 (MZUCR8456, L: 20 mm).

Felimida regalis (Ortea, Caballer & Moro, 2001)
(Fig. 5D)

Synonym

Noumea regalis Ortea, Caballer & Moro, 2001: 2-6, figs. 1-3.

Material

Cahuita National Park (CNP1): 1 specimen (MZUCR8234), L: 6 mm. Subtidal 4 m deep, under a rock.

Remarks

Cahuita is the type locality of *F. regalis* (Ortea et al., 2001: 2). The specimen herein studied fits all the features originally described for this species, but presents less orange-reddish pigment on the dorsum, probably due to its small size.

Felimare ruthae (Ev. Marcus & Hughes, 1974)

Synonym

Hypselodoris ruthae Ev. Marcus & Hughes, 1974: 518-520, figs. 36-40.

Material

Cahuita National Park (CNP1): 4 specimens (MZUCR8214, MZUCR8287, MZUCR8288, MZUCR8289), L: 7-12 mm; off shore Manzanillo (OSM15): 1 specimen (MZUCR 8355), L: 15 mm; RNVS, Gandoca-Manzanillo (PPB14): 1 specimen (MZUCR8361), L: 14 mm; RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8380), L: 14 mm; RNVS, Gandoca-Manzanillo (MR17): 2 specimens (MZUCR8449, MZUCR8450), L: 10 mm each. Subtidal 2-12 m deep, under rocks.

Felimare kempfi (Ev. Marcus, 1971)

Synonym

Chromodoris kempfi Ev. Marcus, 1971: 940-941, figs. 34-38

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8378, MZUCR8379), L: 15-22 mm. Subtidal 6 m deep, together on a rock.

Family Discodorididae Bergh, 1891
Discodoris branneri (MacFarland, 1909)

Synonyms

Discodoris evelinae Er. Marcus, 1955: 153-157; *Discodoris hedgpethi,* Ev. Marcus & Er. Marcus, 1960: 254-256, figs. 7-11.

Material

Cahuita National Park (CNP5): 3 specimens (MZUCR8257, MZUCR8258, MZUCR8251), L: 27-50 mm; Cahuita National Park (CNP9): 2 specimens (MZUCR8293, MZUCR8294), L: 30-45 mm; RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8396, MZUCR8326), L: 24-60 mm; Punta Uva (PU18): 1 specimen (MZUCR8419), L: 35 mm; RNVS, Gandoca-Manzanillo (PPB14): 2 specimens (MZUCR8365, MZUCR8366), L: 25-30 mm. Subtidal 2-7 m deep, under rocks.

Remarks

We prefer not to follow the generic name proposed by Dayrat (2010) for this species (i.e., "*Montereina*" or *Discodorididae*) since it was proposed for a metaphyletic group of many species that cannot be characterized by any diagnostic character (Dayrat, 2010: 221), nor by geographic

distribution. In some cases, proposing temporary names may cause even more nomenclatural problems in taxonomy than acting conservatively. We prefer to continue to use the generic name *Discodoris* for this species until the phylogeny and genus boundaries of Discodorididae are better resolved.

Platydoris angustipes (Mörch, 1863)

Synonyms

Doris (Argus) angustipes Mörch, 1863: 32; *Platydoris angustipes var. alaleta* Bergh, 1877: 505, pl. 58m figs. 13-18; *Platydoris rubra* White, 1952: 118.

Material

Cahuita National Park (CNP5): 1 specimen (MZUCR8229), L: 7 mm; RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8399), L: 9 mm. Subtidal 4-8 m deep, under rocks.

Taringa tritorquis Ortea, Pérez & Llera, 1982

Material

RNVS, Gandoca-Manzanillo (JC16): 1 specimen (MZUCR8435), L: 7 mm; Cahuita National Park (CNP5): 1 specimen (MZUCR8250), L: 6 mm. Subtidal 4 and 25 m deep, under rocks.

Jorunna cf. *spazzola* (Er. Marcus, 1955)

Synonyms

Awuka spazzola Er. Marcus, 1955: 156-158, fig. 180-192; *Jorunna luisae* Ev. Marcus, 1976: 45-50, figs. 33-46.

Material

RNVS, Gandoca-Manzanillo (PM12): 3 specimens (MZUCR8329, MZUCR8330, MZUCR8409), L: 8-14 mm. Subtidal 6-7 m deep, under rocks.

Morphological comments

The background color of the living animals is light gray with several minute dark spots all over the dorsum. The dorsum is covered with caryophyllidia. There are mantle glands around the mantle edge in all of the specimens examined. Rhinophores and gills are light brown, speckled with brown minute spots, with opaque white tips. The gill is spreading. Radular innermost teeth without denticles and outermost teeth pectinated.

Remarks

Although the radular morphology of the Costa Rican specimens is in accordance with the original description of

J. spazzola, some differences exist (concerning dorsal papillae and gill morphology) comparing the Costa Rican specimens with *J. spazzola* specimens from its type locality in southeastern Brazil.

Atagema browni Thompson, 1980

Material

RNVS, Gandoca-Manzanillo (PM12): 3 specimens (MZUCR8331, MZUCR8332, MZUCR8398), L: 10-20 mm. Subtidal 4-7 m deep, under rocks.

Sclerodoris worki (Ev. Marcus & Er. Marcus, 1967)
(Fig. 3A)

Synonym

Anisodoris worki Ev. Marcus & Er. Marcus, 1967: 66-70, figs. 85-89.

Material

RNVS, Gandoca-Manzanillo (PM12): 3 specimens (MZUCR8328, MZUCR8400, MZUCR8401), L: 14-17; Punta Uva (PU18): 1 specimen (MZUCR8434), L: 25 mm. Subtidal 3-7 m deep, under rocks.

Remarks

This species is a new record for Costa Rica.

Diaulula greeleyi (MacFarland, 1909)

Synonym

Peltodoris greeleyi MacFarland, 1909: 84-88, pl. 15, figs. 77-82; *Peltodoris nayarita* Ortea & Llera, 1981: 47-51, figs. 1-4.

Material

Cahuita National Park (CNP5): 1 specimen (MZUCR8216), L: 18 mm. Subtidal 2 m deep, under rocks.

Diaulula hummelincki (Ev. Marcus & Er. Marcus, 1963)
(Fig. 3B)

Synonym

Peltodoris hummelincki Ev. Marcus & Er. Marcus, 1963: 27-30, figs. 32-35.

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8342, MZUCR8343), L: 6-8 mm. Subtidal 7 m deep, under rocks.

Remarks

This species is a new record for Costa Rica, and it has not been reported from other localities west of Aruba (Valdés et al., 2006).

Diaulula phoca **(Ev. Marcus & Er. Marcus, 1963)**
(Fig. 5E)

Synonym

Discodoris phoca Ev. Marcus & Er. Marcus, 1967: 78-80, figs. 99-101.

Material

Cahuita National Park (CNP5): 3 specimens (MZUCR8239, MZUCR8240, MZUCR8298), L: 24-60 mm; RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8327), L: 30 mm. Subtidal 3-7 m deep, under rocks.

Geitodoris pusae **(Er. Marcus, 1955)**

Synonym

Discodoris pusae Er. Marcus, 1955:147-151, fig. 151-165.

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8411, MZUCR8334), L: 10-18 mm. Subtidal 4-7 m deep, under a rock.

Discodorididae sp. 1
(Fig. 3C)

Material

Cahuita National Park (CNP5): 2 specimens (MZUCR8259, MZUCR8260), L: 45-70 mm. Subtidal 2-6.5 m deep, under a rock.

Morphological comments

Body oval. Background color light cream with several irregular light brown spots. A few darker brown spots occur randomly on the dorsum. Dorsum covered with small rounded tubercles. Rhinophores and gill light brown. Gill leaves cream with bright apex. Mantle edge with small, irregular opaque white areas, probably glands. Ventral mantle white, with a few small and irregular bright brown spots. Ventral foot, yellowish.

Remarks

Valdés et al. (2006) illustrated six potential undescribed Discodorididae species from the Caribbean Sea, indicating a large and unknown diversity of the group in the region.

Discodorididae sp. 1 represents one more previoulsy unknown morphotype from the Caribbean Sea. The body shape resembles Discodorididae sp. 3 (Valdés et al., 2006: 192) but the color pattern is different, the latter having a translucent grayish mantle, without the brownish cream pattern of the species herein studied.

Discodorididae sp. 2
(Fig. 5F)

Material

RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8456), L: 20 mm. Subtidal 2 m deep, under a rock.

Morphological comments

Body oval, elongated. Rhinophores large and elongated, gill relatively small. Background color beige with smooth irregular light brown patches. Rhinophores and gill cream, covered in very small dark spots with light cream tips. Mantle edge with small, irregular, opaque white areas, probably glands.

Remarks

As commented above, the Discodorididae diversity in the Caribbean region is largely unknown. Discododoris sp. 2 may represent an unusual form of *Diaulula phoca* or an undescribed species with larger rhinophores and lighter body color.

Family Dendrodorididae O'Donoghue, 1924
Dendrodoris krebsii **(Mörch, 1863)**

Synonym

Doris krebsii Mörch, 1863: 34-35.

Material

Cahuita National Park (CNP1, CNP7, CNP8, CNP9, CNP10): 10 specimens (MZUCR8188 to MZUCR8189, MZUCR8215, MZUCR8249, MZUCR8253 to MZUCR8256, MZUCR8292, MZUCR8297), L: 3-30 mm; RNVS, Gandoca-Manzanillo (MR17): 16 specimens (MZUCR8317 to MZUCR8325, MZUCR8381 to MZUCR8385), L: 4-25 mm; off shore Manzanillo (OSM15): 3 specimens (MZUCR8403, MZUCR8348, MZUCR8349), L: 5-10 mm; RNVS, Gandoca-Manzanillo (PPB14): 1 specimen (MZUCR8367), L: 18 mm; Punta Uva (PU18): 4 specimens (MZUCR8424, MZUCR8425, MZUCR8426, MZUCR8427), L: 7-15 mm. Subtidal 2-13 m deep, most under rocks.

Doriopsilla espinosai Valdés & Ortea, 1998
(Fig. 3D)

Material

Cahuita National Park (CNP9): 1 specimen (MZUCR8291), L: 3.5 mm. Subtidal 4 m deep, over a rock.

Morphological comments

Background color light orange with small red spots irregularly distributed on the dorsum, and small whitish spots near the mantle edge. Rhinophores and gills with the same color as the body. Dorsum covered by spicules and small tubercles. Gill small.

Remarks

Doriopsilla espinosai is a little-studied species previously known only from Cuba (type locality) and the Bahamas. The specimen herein studied has a darker orange body color than the one reported in the original description, probably due to prey color differences. Body shape, rhinophores, gill size and disposition, and small red spots on the notum are characteristics of the species. This species is a new record for Costa Rica.

Doriopsilla pharpa Er. Marcus, 1961
(Fig. 3E)

Synonym

Doriopsilla leia Er. Marcus, 1961: 144-146, figs. 15-18.

Material

Cahuita National Park (CNP5): 1 specimen (MZUCR8244), L: 3 mm. Subtidal 3 m deep, on top of a rock.

Remarks

This species is a new record for Costa Rica, and it has not been reported from other localities west of Cuba (Valdés et al., 2006).

Family Dorididae Rafinesque, 1815
Aphelodoris antillensis Bergh, 1879

Synonym

Doris bistellata Verrill, 1900: 548, pl. 66, fig. 2.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR 8339), L: 8 mm. Subtidal 7 m deep, on top of a rock.

Hexabranchus morsomus Ev. Marcus & Er. Marcus, 1962

Material

Cahuita National Park (CNP1): 5 specimens (MZUCR 8187, MZUCR8227, MZUCR8228, MZUCR8233, MZUCR8248), L: 8-20 mm; RNVS, Gandoca-Manzanillo (PM12): 3 specimens (MZUCR8305, MZUCR8306, MZUCR8388), L: 4-35 mm, 4 specimens (MZUCR8374), L: 2-6 mm; Off shore Manzanillo (OSM15): 1 specimen (MZUCR8350), L: 5 mm; Punta Uva (PU18): 2 specimens (MZUCR8420, MZUCR8421), L: 16-37 mm; Gandoca-Manzanillo (JC16): 1 specimen (MZUCR8438), L: 25 mm; RNVS, Gandoca-Manzanillo (MR17): 2 specimens (MZUCR8451, MZUCR8455), L: 5-10 mm; RNVS, Gandoca-Manzanillo (PPB14): 1 specimen (MZUCR 8362), L: 3 mm. Subtidal 3-15 m deep, under rocks.

CLADOBRANCHIA Willan & Morton, 1984
Family Bornellidae Bergh, 1874
Bornella calcarata Mörch, 1863

Material

Cahuita National Park (CNP9): 1 specimen (MZUCR8186), L: 27 mm; RNVS, Gandoca-Manzanillo (PM12): 3 specimens (MZUCR8301, MZUCR830 MZUCR8304), L: 7-70 mm; RNVS, Gandoca-Manzanillo (MR17): 3 specimens (MZUCR8445, MZUCR8446, MZUCR8447), L: 31-65 mm.

Remarks

Subtidal 6.5-12.5 m deep, on top of rocks.

Family Dotidae Gray, 1853
Doto duao Ortea, 2001

Material

Cahuita National Park (CNP1): 3 specimens (MZUCR8204, MZUCR8205, MZUCR8206), L: 2-3 mm. Subtidal 12.5 m deep, on hydrozoans.

Family Tritoniidae Lamarck, 1809
Tritonia cf. *pickensi* Ev. Marcus & Er. Marcus, 1967
(Fig. 3F)

Material

Cahuita National Park (CNP10): 1 specimen (MZUCR8300), L: 2 mm. Subtidal 4 m deep, under a rock.

Morphological comments

Background color translucent white with an opaque white line located at the center of the dorsum that runs just behind

the rhinophores, and ends before the end of the foot. This line takes the form of two or three opaque white diamonds and joins at the base of each gill. The body is scattered with minute opaque white and blue dots. Cerata are very short, single or bifurcated. The edge of the oral veil has two short appendages.

Remarks

Our specimen shares several morphological characters with *T. pickensi*, originally described from the Gulf of California, such as body morphology and color pattern, including an opaque white band that spans its length. The color of the intestinal gland and number of velar appendages and processes of our specimen do not match the description for *T. pickensi*, but it could be due to intra-specific variation and specimen size. The occurrence of *T. pickensi* at the Caribbean coast of Costa Rica needs to be confirmed by further comparative studies. This species has been reported from Baja California Sur, Mexico down to the Pacific coast of Costa Rica and Panama (Camacho et al., 2005). This is the first record in the Atlantic Ocean.

Family Aeolidiidae Gray, 1827
Berghia creutzbergi Er. Marcus & Ev. Marcus, 1970

Synonyms

Spurilla creutzbergi (Er. Marcus & Ev. Marcus, 1970): 87, figs. 145-147, Rudman (1982): 164; *Millereolidia ritmica* (Ortea, Caballer & Espinosa, 2003): 133-137, figs. 2, 4B, pl. 1, fig. B.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8340), L: 15 mm. Subtidal 7 m deep, under a rock.

Remarks

The genus *Berghia* has been considered a junior synonym of *Spurilla* by some authors (Rudman, 1982; García-Gómez & Thompson, 1990). Recently, Carmona et al. (2013) confirmed that *Berghia* is a valid genus.

Limenandra nodosa Haefelfinger & Stamm, 1958
(Fig. 3G)

Synonym

Baeolidia nodosa (Haefelfinger & Stamm, 1958): 418-423, fig. 1, Gosliner (1980), 66-69, fig. 19.

Material

RNVS, Gandoca-Manzanillo (PM12): 1 specimen (MZUCR8344), L: 4 mm. Subtidal 7 m deep, on *Sargassum*.

Remarks

This species is a new record for Costa Rica.

Family Facelinidae Bergh, 1889
Nanuca sebastiani Er. Marcus, 1957

Material

Cahuita National Park (CNP5): 1 specimen (MZUCR8242), L: 3 mm. Subtidal 3 m deep, on *Sargassum*.

Phidiana lynceus Bergh, 1867

Synonyms

Phidiana selencae Bergh, 1879: 560-563, pl. 6, figs. 10-18; *Phidiana brevicauda* Engel, 1925: 35-38, figs .4-6; *Phidiana adiuncta* Ortea, Caballer & Moro, 2004: 86-94, fig. 3, pl. 1, figs. E-F.

Material

Cahuita National Park (CNP2, CNP5, CNP7): 7 specimens (MZUCR8210-MZUCR8213, MZUCR8238, MZUCR8241, MZUCR8247), L: 3-18 mm; RNVS, Gandoca-Manzanillo (PM12): 5 specimens (MZUCR8311, MZUCR8312, MZUCR8313, MZUCR8375, MZUCR8387), L: 10-15 mm; RNVS, Gandoca-Manzanillo (PPB14): 2 specimens (MZUCR8359, MZUCR8360), L: 12-15 mm; Punta Uva (PU18): 1 specimen (MZUCR8422), L: 22 mm; RNVS, Gandoca-Manzanillo (MR17): 1 specimen (MZUCR8457), L: 3 mm. Subtidal 2-7 m deep, under rocks.

Learchis evelinae Edmunds & Just, 1983
(Fig. 3H)

Material

Cahuita National Park (CNP7): 2 specimens (MZUCR8277, MZUCR8278), L: 5 mm each. Subtidal 4 m deep, all specimens found on *Sargassum*.

Remarks

This species is little known. The small specimens herein studied fit in part to the color description of the type-material, from Barbados (Edmunds & Just, 1983). Other specimens, from Belize and Martinique, illustrated in Valdés et al. (2006) also have differences in color and number of cerata. M. Edmunds (pers. communication) corroborated our identification. This species is a new record for Costa Rica.

Family Flabellinidae Bergh, 1889
***Flabellina dana* Millen & Hamann, 2006**

Material

RNVS, Gandoca-Manzanillo (PM12): 2 specimens (MZUCR8389, MZUCR8390), L: 9 mm each. Subtidal 4 m deep, under rocks.

Remarks

Valdés et al. (2006) recorded this species from Costa Rica as *Flabellina* sp. 3.

***Flabellina dushia* (Ev. Marcus & Er. Marcus, 1963)**
(Fig. 3I)

Synonyms

Coryphella dushia Ev. Marcus & Er. Marcus, 1963: 41-42, figs. 52-54.

Material

Cahuita National Park (PV3): 1 specimen (MZUCR8219), L: 7 mm; Cahuita National Park (CNP8): 1 specimen (MZUCR8252), L: 4 mm. Subtidal 2-5.5 m deep.

Remarks

This species is a new record for Costa Rica, and it has not been reported from other localities west of Jamaica.

Discussion

The present study updates and refines the knowledge on the opisthobranch fauna of the Caribbean coast of Costa Rica, including color photographs of uncommon and also previously unknown species. For some species, color information was known from only 1-3 different localities throughout their geographic distributions and different color patterns have been reported among different localities in the Caribbean (Valdés et al., 2006). Hence, our images add to the documented color variation, being part of one goal of this study: provide more data to complement future comparative, molecular studies in order to understand better the limits between intraspecific variation and inter-specific differences.

Recent studies have already indicated that our traditional concepts on some Caribbean 'species' were misleading, with the detection of cryptic species (*Elysia timida*; Carmona et al., 2011, Krug et al., 2012; *Navanax aenigmaticus*; Ornelas-Gatdula et al., 2012), sympatric but very different color morphotypes being the same species (*Chelidonura berolina*; Ornelas-Gatdula et al., 2011), and pairs of species with overlapping body color patterns (*Philinopsis pusa*; Ornelas-Gatdula & Valdés, 2012). These results highlight the importance of obtaining more detailed data on Caribbean species to better understand the opisthobranch fauna of the region.

Besides the need to clarify currently documented species, the Caribbean is still a source of unexplored biodiversity. The Discodorididae is a good example of how much there is yet to be discovered in the region. Recently, at least 11 unknown morphotypes were identified (Valdés et al., 2006; present study) in this group alone, and there are undescribed species known from almost all opisthobranch groups, including Sacoglossa, Pleurobranchomorpha, Chromodorididae, Polyceridae, Facelinidae, and Flabellinidae (Valdés et al., 2006; present study).

In conclusion, we surveyed only three main locales of the southern Costa Rican Caribbean, and found undescribed species as well as many new records. In addition, we identified several potential species complexes. Hence, future surveys should be undertaken using different collecting techniques along with more collecting effort, nighttime surveys, and the exploration of new sites (among other strategies). Future studies should include as much detailed information as possible (i.e., internal and external morphology, histology, genetics, behavior, etc.), including photos of species from different regions of their respective geographic distributions. Only then will be possible to better understand the biodiversity of the Caribbean opisthobranch fauna.

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3. RESULTS

<u>Chapter 4</u>

Padula V, Wirtz P, Schrödl M (2014) Heterobranch sea slugs (Mollusca: Gastropoda) from Ascension Island, South Atlantic Ocean. *Journal of the Marine Biological Association of the United Kingdom.*

Heterobranch sea slugs (Mollusca: Gastropoda) from Ascension Island, South Atlantic Ocean

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The small volcanic island of Ascension is situated in the middle of the South Atlantic Ocean, more than 1500 km from the coast of Africa, its nearest continental area. To date, eight 'opisthobranch' species were reported from the island. As a result of a recent survey, 10 species were found. Seven species are new records from Ascension: Platydoris angustipes *(Mörch, 1863),* Diaulula sp., Dolabrifera dolabrifera *(Rang, 1828),* Aplysia parvula *Guilding in Mörch, 1863 and* Caliphylla mediterranea *A. Costa, 1867, and two new species:* Phidiana mimica *sp. nov.; and* Felimida atlantica *sp. nov. Half of the species found have a wide geographical distribution, being not restricted to the Atlantic Ocean. However, traditional taxonomy based on few characters is probably masking complexes of species.*

INTRODUCTION

Ascension is a small volcanic island situated in the middle of the South Atlantic Ocean. Its nearest land areas are more than 1000 km away (St Helena Island: 1130 km; Liberia, West Africa: 1536 km; Fernando de Noronha archipelago: 2048 km). The approximately triangular island is only 97 km², being surrounded mostly by rocky shores and small sandy beaches. For a description of general characteristics of the coast of Ascension see Price & John (1980). As pointed out by these authors, in the beginning of the 20th Century many expeditions used Ascension as a stop-off point during their journeys. However, few observations were made about the marine life of the island. Improvement on the knowledge came later, with publication of annotated lists of species, in particular those by Rosewater (1975) for marine molluscs, Manning & Chace Jr (1990) for decapod and stomatopod crustaceans and Lubbock (1980) for shore fish.

Rosewater's (1975) list of molluscs included data previously reported by other authors, such as Packer (1968) and mostly Smith (1890a, b), and the information provided by the examination of collections made by Mrs Hutchfield and by R.B. Manning. Rosewater (1975) also considered valid the presence of some species on the island based only on verbal communications by Mrs Hutchfield, that is, without the existence of specimens for examination (e.g. *Cypraea tigris* Linnaeus, 1758 and *Tonna galea* Linnaeus, 1758). His list covered 89 species, eight of them under the 'Opisthobranchia' (Rosewater, 1975: p. 24), including one pyramidellid. The list also included two

species of Siphonariidae. In the past, the family Pyramidellidae was considered part of Opisthobranchia by some authors (e.g. Boettger, 1955) but later included among the group of basal, not well-resolved heterobranchs (Haszprunar, 1985). It is now clear that Opisthobranchia *per se* is not a natural group (Jörger *et al.*, 2010; Kocot *et al.*, 2013, among others), and a recent reclassification of traditional groups such as Acteonoidea, Nudibranchia and Sacoglossa has been presented by Wägele *et al.* (2014).

Among the eight opisthobranch species reported from Ascension by Rosewater (1975), most are shelled forms, including two deep water species collected by 'The Challenger' (see Table 1). No representatives of other diverse groups, such as Nudibranchia or Sacoglossa, were known from the island until now. Based on material collected in a recent expedition by the Shallow Marine Surveys Group (SMSG) and the South Atlantic Environmental Research Institute (SAERI), we here update the information on the heterobranch sea slugs of Ascension Island, including the description of two new species.

MATERIALS AND METHODS

Material was collected manually from tide pools and through SCUBA diving down to a depth of 15 m, in August–September 2012. Specimens were photographed alive, preserved in 96% ethanol and deposited in the malacological collection of the Zoologische Staatssammlung München (ZSM), Germany. Taxonomic identifications were based mostly on external characters, such as body morphology and colour pattern, in comparison to field guides and checklists (Cervera *et al.*, 2004; Valdés *et al.*, 2006), and original descriptions. At least two species presented an external morphology and colour pattern previously unknown for any described species. Specimens of

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Table 1. Heterobranch sea slugs from Ascension. New records marked* (Eupulmonata not included).

Taxon	Record from Ascension	Geographical distribution
"LOWER HETEROBRANCHIA"		
ACTEONOIDEA		
Micromelo undatus (Bruguière, 1792)	Rosewater (1975: p. 24); present study (ZSM Mol 20130108)	Circumglobal
NUDIPLEURA		
PLEUROBRANCHOIDEA		
Pleurobranchus areolatus Mörch, 1863	Rosewater (1975: p. 25); present study (ZSM Mol 20130103, 20130104, 20130113)	Tropical eastern Pacific, western and eastern Atlantic; Ascension
NUDIBRANCHIA		
* *Platydoris angustipes* (Mörch, 1863)	Present study (ZSM Mol 20130105)	Florida to south-eastern Brazil; Ascension*
* *Diaulula* sp.	Present study (ZSM Mol 20130107)	Ascension*
* *Felimida atlantica* sp. nov.	Present study (ZSM Mol 20130114)	Ascension*
* *Phidiana mimica* sp. nov.	Present study (ZSM Mol 20130109, 20130110)	Ascension*
EUOPISTHOBRANCHIA		
UMBRACULOIDEA		
Umbraculum umbraculum (Lightfoot, 1786)	Rosewater (1975: p. 25, as *U. mediterraneum*); present study	Circumglobal
CEPHALASPIDEA		
Cylichna cylindracea (Pennant, 1777)	Smith (1890b)	Eastern Atlantic, Mediterranean Sea, Cape Verde, Canary Islands, Ascension (deep water), St Helena, Tristan da Cunha.
Cylichna orycta (Watson, 1883)	Smith (1890b)	Ascension (deep water)
Haminoea hydatis (Linnaeus, 1758)	Smith (1890a, b); Rosewater (1975: p. 25)	Northeastern Atlantic, Azores, Mediterranean Sea, Ascension and St Helena
ANASPIDEA		
Aplysia dactylomela Rang, 1828	Rosewater (1975: p. 25)	Atlantic and Mediterranean
* *Aplysia parvula* Guilding in Mörch, 1863	Present study (ZSM Mol 20130115)	Circumglobal
* *Dolabrifera dolabrifera* (Rang, 1828)	Present study (ZSM Mol 20130106, 20130112)	Circumglobal
PANPULMONATA		
SACOGLOSSA		
* *Caliphylla mediterranea* A. Costa, 1867	Present study (ZSM Mol 20130111)	Mediterranean Sea, Senegal, Caribbean Sea, Brazil, Ascension*
SIPHONARIOIDEA		
Siphonaria alternata (Say, 1826)	Rosewater (1975: p. 24, as *S. picta*)	Bermuda, Florida, Caribbean Sea, Brazil, Ascension (?)
Williamia gussoni (Costa O. G., 1829)	Smith (1890a, b); Rosewater (1975: p. 24)	Azores, Cape Verde, Mediterranea Sea, St Helena and Ascension
PYRAMIDELLOIDEA		
Pyramidella dolabrata (Linnaeus, 1758)	Rosewater (1957: p. 24)	Circumtropical

these species were dissected under a stereomicroscope. The buccal bulb was manually cleaned and immersed in a solution of 10% sodium hydroxide (NaOH) to dissolve soft tissues. Cleaned jaws and radula were transferred to distilled water and mounted for photography in the scanning electronic microscope LEO 1430VP, at the ZSM. For the study of the reproductive system, it was first cleaned and isolated from adjacent systems and then drawn with the aid of a camera lucida.

RESULTS

During the expedition, ten species were collected: *Micromelo undatus* (Bruguière, 1792); *Pleurobranchus areolatus* Mörch, 1863; *Platydoris angustipes* (Mörch, 1863); *Diaulula* sp.; *Felimida atlantica* sp. nov.; *Phidiana mimica* sp. nov.; *Umbraculum umbraculum* (Lightfoot, 1786); *Dolabrifera dolabrifera* (Rang, 1828); *Aplysia parvula* Guilding in Mörch, 1863; and *Caliphylla mediterranea* A. Costa, 1867. Seven species represent new records for Ascension Island (see species remarks below), among them two new nudibranch species, representing the first record of this group from the island.

<div align="center">

SYSTEMATICS
Class GASTROPODA Cuvier, 1795
HETEROBRANCHIA Gray, 1840
ACTEONOIDEA d'Orbigny, 1843
Family APLUSTRIDAE Gray, 1847
Genus *Micromelo* Pilsbry, 1895
Micromelo undatus (Bruguière, 1792)
(Figure 1A)

</div>

MATERIAL EXAMINED
One specimen, 6 mm long (preserved) (crawling on a rock in 5 m depth, English Bay, Ascension Island) (ZSM Mol 20130108), P. Wirtz coll., 10 September 2012.

REMARKS
Micromelo undatus is considered a circumtropical species (Valdés *et al.*, 2006), but this needs to be tested through
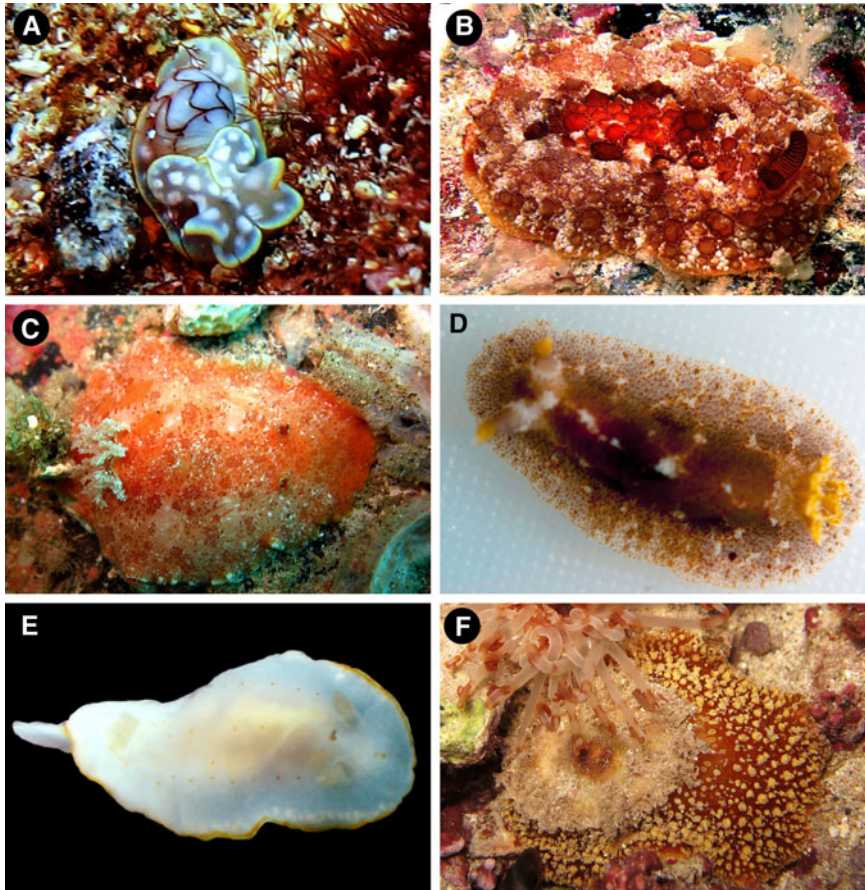
Fig. 1. Heterobranch sea slugs from Ascension Island: (A) *Micromelo undatus* (Bruguière, 1792) (ZSM Mol 20130107); (B) *Pleurobranchus areolatus* Mörch, 1863 (ZSM Mol 20130104); (C) *Platydoris angustipes* (Mörch, 1863) (ZSM Mol 20130105); (D) *Diaulula* sp. (ZSM Mol 20130107, photograph by Simon Morley); (E) *Felimida atlantica* sp. nov. (holotype, ZSM Mol 20130114); (F) *Umbraculum umbraculum* (Lightfoot, 1786) (not collected).

comparative morphological and molecular studies. It was first recorded from Ascension by Rosewater (1975) based on four specimens, one of them collected alive.

<div align="center">

EUTHYNEURA Spengel, 1881
NUDIPLEURA Wägele & Willan, 2000
PLEUROBRANCHOIDEA Gray, 1827
Family PLEUROBRANCHIDAE Gray, 1827
Genus *Pleurobranchus* Cuvier, 1804
Pleurobranchus areolatus Mörch, 1863
(Figure 1B)

</div>

MATERIAL EXAMINED
Three specimens, 30 mm long preserved (ZSM Mol 20130103), 32 mm long preserved (ZSM Mol 20130104) and 25 mm long preserved (ZSM Mol 20130113) (found under rocks between 5 and 10 m depth, English Bay and Soudan Bay), coll. P. Wirtz, August–September 2012.

REMARKS
Originally described from St Thomas, in the Caribbean Sea (Mörch, 1863), *P. areolatus* was later recorded from many localities in the tropical western Atlantic (see Valdés *et al.*, 2006), and also in the eastern Pacific and eastern Atlantic (Cervera *et al.*, 2004; Camacho-García *et al.*, 2005). The first record of this species from Ascension was provided by Rosewater (1975) based on a single specimen. It is not clear

if the differences in body colour pattern and dorsal papillae reported for *P. areolatus* may, in fact, be indicative that more than one species is involved (Rudman, 2000). Ascension specimens present the most common reddish pattern known for the species, being very similar to the specimens from Brazil, illustrated by García *et al.* (2002: Figure 2H) and Padula *et al.* (2012, Figure 5D).

<div align="center">

NUDIBRANCHIA Cuvier, 1817
EUCTENIDIACEA Tardy, 1970
Family DISCODORIDIDAE Bergh, 1891
Genus *Platydoris* Bergh, 1877
Platydoris angustipes (Mörch, 1863)
(Figure 1C)

</div>

MATERIAL EXAMINED
Two specimens, 35 and 42 mm long preserved (under a rock in 10 m depth, English Bay) (ZSM Mol 20130105), coll. P. Wirtz, 6 September 2012.

REMARKS
Widespread in the tropical western Atlantic, with many records along the Caribbean to south-eastern Brazil (Valdés *et al.*, 2006; Padula *et al.*, 2012), *P. angustipes* is herein for the first time recorded from Ascension Island, representing the easternmost known record for the species.

Genus *Diaulula* Bergh, 1878
Diaulula sp.
(Figure 1D)

MATERIAL EXAMINED

One specimen, 22 mm long preserved (under a rock in 10 m depth, North East Bay) (ZSM Mol 20130107), coll. S. Morley, 31 August 2012.

REMARKS

Due to the high number of similar morphotypes or species, the family Discodorididae represents one of the most puzzling groups in the Atlantic Ocean (Camacho-García *et al.*, 2014). This morphotype from Ascension resembles *Diaulula hummelincki* (Ev. Marcus & Er. Marcus, 1963) in the colour of the rhinophores and gill, and young specimens of *Discodoris branneri* MacFarland, 1909 in general colour and external morphology (Alvim & Pimenta, 2013). The specific identity of this material can only be clarified after further comparative morphological and molecular studies.

Family CHROMODORIDIDAE Bergh, 1891
Genus *Felimida* Ev. Marcus, 1971
Felimida atlantica sp. nov.
(Figures 1E, 2, 6A)

TYPE MATERIAL

Holotype: 12 mm long, preserved. Dissected, radula and jaws mounted on stub for SEM, reproductive system studied (English Bay, water depth: 15 m, under a large rock) (ZSM Mol 20130114), coll. P. Wirtz, 9 September 2012.

EXTERNAL MORPHOLOGY

Body long, oval in shape, flattened. Mantle smooth, with a series of densely arranged, small, irregular granular glands (mantle dermal formations, 'MDFs') along its border, except on the anterior margin. Foot relatively straight, posterior region projected and pointed. Oral tentacles short and conical. Rhinophoral sheath low, base of rhinophores smooth, distal portion with 15 adjacent lamellae. Gill relatively short, with five unipinnate leaves.

BODY COLOUR

Body predominantly white or translucent white. Dorsal mantle with three longitudinal series of small orange dots, running from each rhinophore and from the midpoint between them in the direction of the gill. Mantle edge bordered by a thin yellowish orange band. Rhinophores and gill whitish, pale cream (Figure 1E). Ventral mantle and foot white.

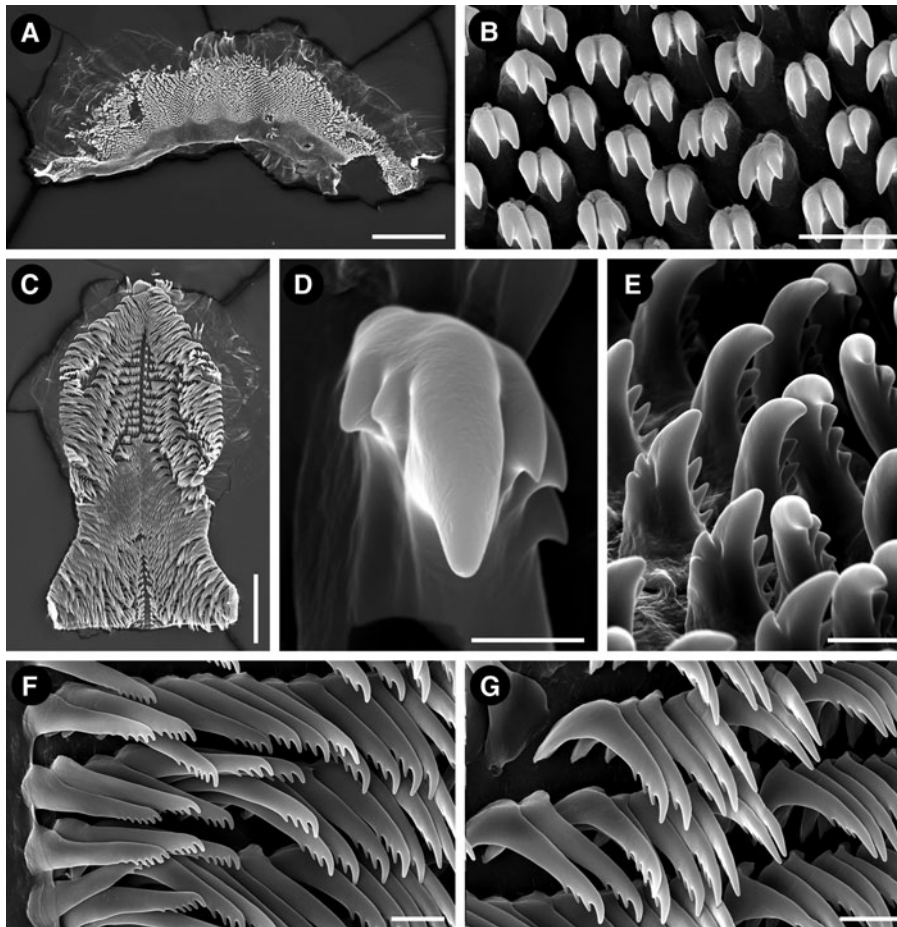
Fig. 2. *Felimida atlantica* sp. nov. (holotype, ZSM 20130114), SEM micrographs. Labial cuticle: (A) opened labial cuticle; (B) elements of the labial cuticle. Radula: (C) entire radula; (D) detail of the first lateral tooth; (E) first lateral teeth, ventral view; (F) outermost lateral teeth; (G) mid-lateral teeth. Scale bars: A, 200 μm; B, 10 μm; C, 200 μm; D, 5 μm; E, 10 μm; F, G, 20 μm.

LABIAL CUTICLE AND RADULA

Oral tube initially narrow, widening posteriorly in the junction with the labial cuticle; when open, labial cuticle is irregular, semi-oval in shape (Figure 2A). Labial cuticle covered by many small, mostly bicuspid elements. In some elements, each cusp may be subdivided, presenting a total of three or four cusps (Figure 2B). Radula wider in its posterior portion; posterior end straight (Figure 2C). Radular formula 42 × 26.0.26 in the 12 mm fixed holotype (ZSM Mol 20130114). First lateral tooth with a large base and a prominent central cusp; up to three external short and triangular cusps disposed in series; one or two short internal cusps near central one. Lateral teeth thin and elongated, with a series of apical, small, rounded cusps; four in the first laterals and six in the most external ones. Outermost lateral teeth straighter than lateral ones, with a shorter base and up to seven apical, small, rounded cusps (Figures 2D–G).

REPRODUCTIVE SYSTEM

Hermaphroditic, triaulic, anterior portion occupying a relatively small space between the buccal mass and digestive gland. Hermaphrodite duct wide, flattened and short; ampulla moderately long, thin, situated above the female gland. Prostate elongated, with many folds; distal deferent duct wide and folded, disposed laterally and ventrally to seminal receptacle; transition between deferent duct and penis well demarcated; male and female atrium in a common space. Vagina very thin and long, projected below to the seminal receptacle; seminal receptacle cylindrical, 1/2 of bursa size, inserting ventrally in the vagina region through a small and curved region; bursa copulatrix rounded. Uterine duct long, thin, resembling vagina, projecting ventrally from bursa copulatrix in direction to the gonopore, inserting female gland mass near to oviduct. Oviduct short. Female glands well developed, nidamental region with a rounded portion, ventrally to vagina (Figure 6A).

ETYMOLOGY

The specific name refers to the Atlantic Ocean.

GEOGRAPHIC DISTRIBUTION

Only known from its type locality: English Bay, Ascension Island, South Atlantic Ocean.

REMARKS

Due to the body form, smooth mantle, the arrangement of MDFs, pectinate radular teeth and the arrangement of the reproductive system, the single specimen studied is allocated in the genus *Felimida*. According to Johnson & Gosliner's hypothesis (2012) *Felimida* comprises Atlantic species previously attributed to the genera *Chromodoris* and *Glossodoris*. *Felimida atlantica* sp. nov. resembles four other Atlantic and Mediterranean *Felimida* species: *Felimida grahami* (Thompson, 1980) and *Felimida paulomarcioi* (Domínguez, García & Troncoso, 2006) from the Caribbean Sea and Brazil, respectively, *Felimida kpone* (Edmunds, 1981) from Ghana, and *Felimida purpurea* (Risso in Guérin, 1831), from the Mediterranean and the eastern Atlantic. All these species share the general whitish dorsal mantle, with or without orange/pink spots or lines, with a marginal yellowish orange line, and purple/reddish pigment in the rhinophores and branchial leaves (Edmunds, 1981; Debelius & Kuiter,

2008). Also, *F. purpurea* and *F. paulomarcioi* share a very similar reproductive system (García-Gomez, 2002; Domínguez *et al.*, 2006), while the reproductive system of *F. grahami* and *F. kpone* were not described up to date. These species are differentiated by details in coloration and radular morphology. *Felimida atlantica* sp. nov differs from all by having white rhinophores and gill. This is an important feature because the colour of these structures does not present wide variation in *Felimida* species. Differences in the radular morphology are more difficult to state because the general pattern in *Felimida* is very similar. Few specimens of each *Felimida* species were anatomically studied and potential intraspecific variation, including ontogenetic, is not well known. However, the innermost lateral teeth seem to carry some specific information. Innermost lateral teeth of *Felimida atlantica* sp. nov. have one, mostly two, internal and only three external cusps (Figure 2D, E). This agrees with the variation reported for *F. purpurea*, two internal, three–four external cusps (García-Gomez, 2002), but these species differ in the colour of the rhinophores and the gill and also in the dorsal colouration, *F. purpurea* not presenting the small orange dots found in *F. atlantica* sp. nov. The dorsal pattern of *F. atlantica* sp. nov. agrees with the description of *F. grahami*, *F. paulomarcioi* and *F. kpone*, with orange/reddish spots disposed in longitudinal lines. In fact, it is not clear if *F. paulomarcioi* does not simply represent a variation of *F. grahami*, as commented by Padula *et al.* (2011). *Felimida atlantica* sp. nov. differs from these and other known *Felimida* species by the diagnostic white rhinophores and gill, combined with the reduced number of cusps in the innermost lateral teeth.

DEXIARCHIA Schrödl, Wägele & Willan, 2001
Family FACELINIDAE Bergh, 1889
Genus *Phidiana* Ev. Marcus, 1971
Phidiana mimica sp. nov.
(Figures 3A, B, 4, 5, 6B)

TYPE MATERIAL

Holotype: 4 mm long preserved. Dissected, radula and jaws mounted on stub for SEM, reproductive system not studied (English Bay, water depth: 10–15 m, under a rock) (ZSM Mol 20130110), coll. P. Wirtz, 9 September 2012.

Paratype: 7 mm long preserved. Dissected, radula and jaws mounted on stub for SEM, reproductive system studied (English Bay, water depth: 10–15 m, under a rock) (ZSM Mol 20130109), coll. P. Wirtz, 9 September 2012.

EXTERNAL MORPHOLOGY

Body long and narrow, distinct and elongated head with long and pointed oral tentacles; rhinophores comparatively short and smooth. Foot with the same width as the body, except the anterior portion that is wider. Anterior region of the foot curved with thin lateral projections; posterior end short and pointed. Cylindrical and elongated cerata distributed in groups; anterior group forming a short arch, posterior groups in lines; each group with 8–15 cerata. Anus situated at the base of the anterior cerata of the second ceratal cluster, on the right side of the body. Genital aperture situated laterally on the right side of the body below the first group of cerata. The position of the renal pore could not be determined.
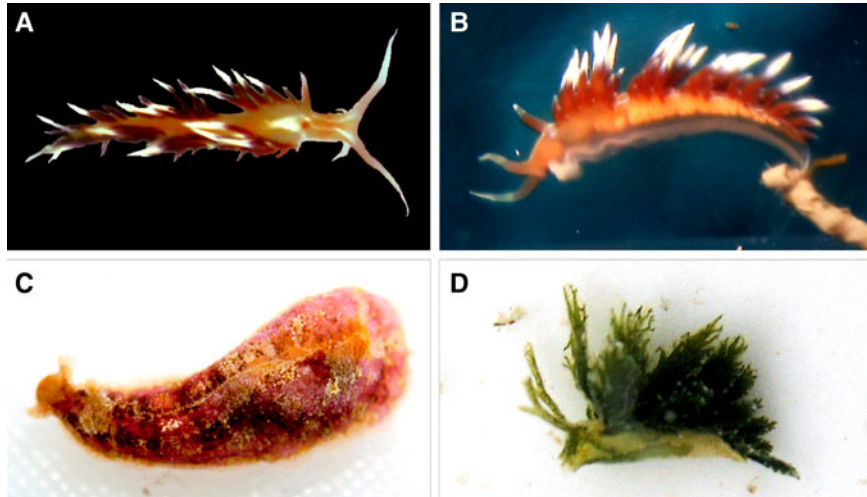
Fig. 3. Heterobranch sea slugs from Ascension Island: (A) *Phidiana mimica* sp. nov., dorsal view (holotype, ZSM Mol 20130109); (B) *Phidiana mimica* sp. nov., lateral view (holotype, ZSM Mol 20130109); (C) *Dolabrifera dolabrifera* (Rang, 1828) (ZSM Mol 20130112, photograph by Simon Morley); (D) *Caliphylla mediterranea* A. Costa, 1867 (ZSM 20130111).

BODY COLOUR

Body orange; dorsally, from the base of each oral tentacle a thin dorsal white line runs in direction to the region between the rhinopores. Dorsal region above the pericardium with a triangular white spot. Dorsal region posterior to the pericardium orange. Foot corners translucent white. Oral tentacles with orange-reddish bases, central region fade yellowish and distal portion white. Rhinophores reddish with white tips. Cerata deep red in their lower half, with a short bluish zone in transition to the white distal portion (Figure 3A, B). It seems that the deep red colour of the cerata is derived from the content of the digestive gland, but it could not be confirmed through the photographs or examining the preserved material. The bluish zone and the distal white portion are pigments on the surface of the cerata.

JAWS AND RADULA

Uniseriate radula with 17 (holotype ZSM Mol 20130110) and 19 teeth (paratype ZSM Mol 20130109); radular teeth with a prominent and smooth central cusp (Figure 4D) and five or six lateral smooth denticles (Figure 4E). The paratype is a teratological specimen with asymmetrical teeth. Some teeth present one side with few (3–6) large denticles and the other with up to 20 small denticles or even smooth (Figure 5B).
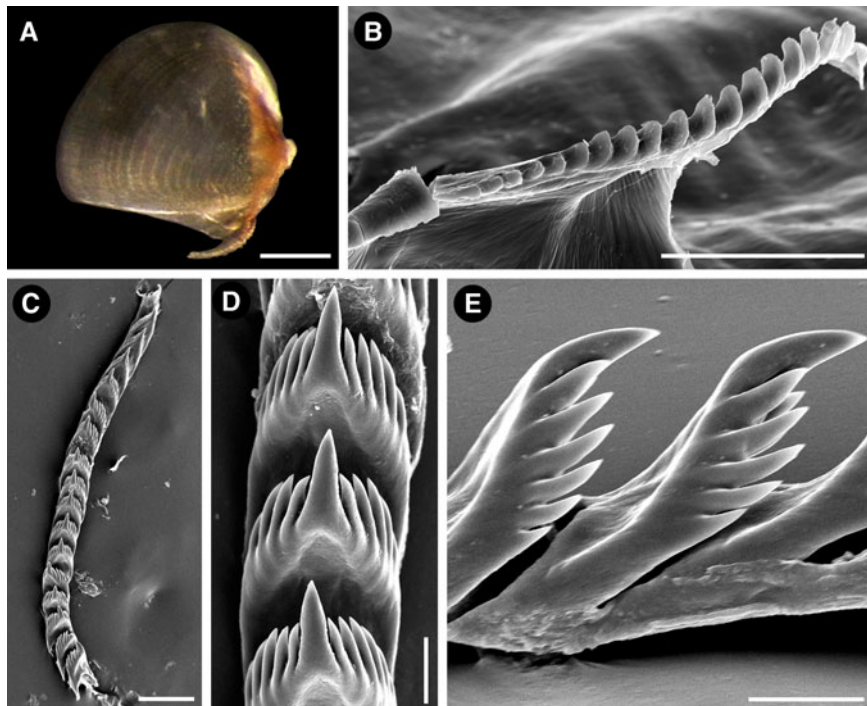
Fig. 4. *Phidiana mimica* sp. nov.: (A) left jaw (photograph on stereo microscope) (paratype, ZSM 20130109). SEM micrographs: (B) border of the jaw (paratype, ZSM 20130109); (C) entire radula (holotype, ZSM 20130110); (D) radular teeth (holotype, ZSM 20130110); (E) detail of radular teeth (holotype, ZSM 20130110). Scale bars: A, 250 μm; B, C 100 μm; D, E, 20 μm.
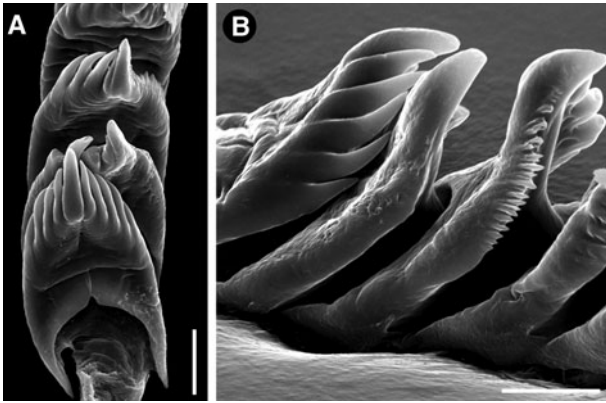
Fig. 5. *Phidiana mimica* sp. nov., teratological radula of the paratype (ZSM 20130109): (A) radular teeth, dorsal view; (B) lateral view of radular teeth showing teratology. Scale bars: A, 25 μm; B, 20 μm.

Holotype and paratype with thin and relatively high jaws (Figure 4A). Masticatory border of the jaws long, projected and denticulate, with a single row of large and spaced, spoon-like teeth (Figure 4A, B).

REPRODUCTIVE SYSTEM

Hermaphroditic, androdiaulic. Proximal gonoduct thin and long. Ampulla elongated, wide, with a turn on its proximal region. Distal portion of ampulla narrowing, postampullary gonoduct short, dividing into deferent duct and oviduct. A differentiate prostate not present. Deferent duct very short, thin, connected to a curved, muscular, penis. A small projection is present in the superior portion of the penis, appearing as a small protuberance above the gonopore (Figure 6B). Vagina elongated, moderately wide, connecting to the female gland mass, oviduct and the stalk of receptaculum seminis; the latter with irregular shape and surface, having a distinct yellowish colour.

ETYMOLOGY

From the Latin *mimicus* (and the Greek *mimikos*) due to the similarity in external appearance of the new species to *Phidiana lynceus* Bergh, 1867.

GEOGRAPHICAL DISTRIBUTION

Only known from its type locality: English Bay, Ascension Island, South Atlantic Ocean.

REMARKS

At first glance, based on general body morphology and colour, the studied specimens could erroneously be identified as belonging to *Phidiana lynceus*, a common tropical western Atlantic species, recorded also from the Canary Islands and Ghana (Edmunds, 1975; Cervera *et al.*, 2004). However, there are differences in the shape of the rhinophores, being lamellated in *P. lynceus* and smooth in *P. mimica* sp. nov., and in the colour of some regions of the body, such as the rhinophores and dorsal region, posterior to the head. The rhinophores of *P. lynceus* have transparent bases, the central region orange or red and a yellowish distal portion (Valdés *et al.*, 2006: p. 257), while they are reddish with white tips in *P. mimica* sp. nov. A central longitudinal white line runs along the entire dorsal region in *P. lynceus*, while in *P. mimica* sp. nov. it is absent. The jaws and the radula of the two species are also different: the jaws of *P. lynceus* are proportionally longer than the jaws of *P. mimica* sp. nov.; *P. lynceus* has 6–9 denticles on each side of the prominent central cusp of the radular teeth (Bergh, 1867; Padula, 2007), while the central cusp is smooth in the teeth of *P. mimica* sp. nov.

Another species that resembles *P. mimica* sp. nov. is *Phidiana indica* (Bergh, 1896) from the tropical Indo-Pacific but also recorded in the Mediterranean as an exotic species (Zenetos *et al.*, 2003). However, the general body colour pattern is different, *P. indica* presenting blue and yellow areas on the oral tentacles and cerata which are absent in *P. mimica* sp. nov.

As pointed recently in a broad molecular phylogenetic study in aeolids (Carmona *et al.*, 2013: figure 1), the traditional generic placements in the family Facelinidae seem to not reflect the natural history of the group and apparently need a general revision. We tentatively allocated the new species in the genus *Phidiana* based on characteristics of external morphology, jaw, radula and reproductive system (Rudman, 1980, 1999). An exception is the smooth rhinophores, as most *Phidiana* species have perfoliated rhinopores, but *P. indica*, for example, does not. This species formerly was
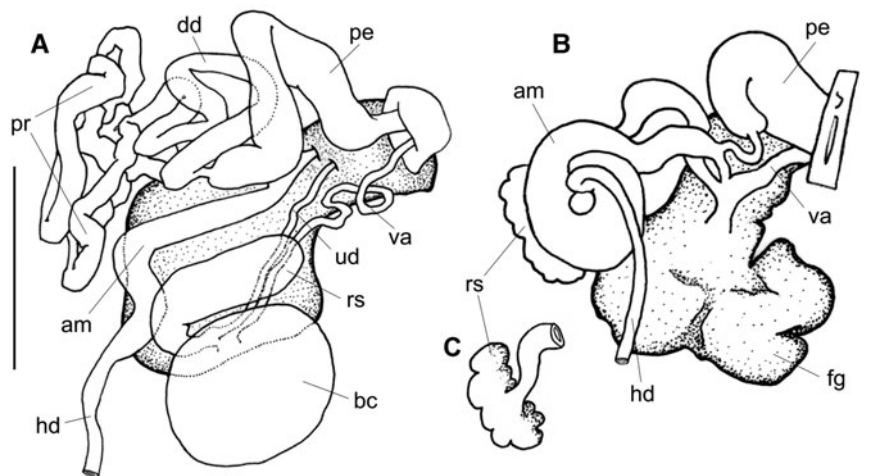

Fig. 6. Reproductive system, dorsal view: (A) *Felimida atlantica* sp. nov. (holotype, ZSM 20130114); (B) *Phidiana mimica* sp. nov. (holotype, ZSM 20130109). Scale bars: A, 1 mm; B, 0.5 mm. am, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland; hd, hermaphrodite duct; pe, penis; pr, prostate; rs, receptaculum seminis; ud, uterine duct; va, vagina.

placed into the genera *Learchis*, *Caloria*, *Hervia* and *Facelina* by different authors (see Rudman, 1999).

One unusual feature of the paratype of *P. mimica* sp. nov. is the asymmetrical morphology of the radular teeth, with a single tooth presenting different shapes on each side of the central cusp, and adjacent teeth presenting different morphology (Figure 5B). It probably represents an abnormality, indeed illustrates how the systematics of aeolid nudibranchs can be even more complicated. Only new comprehensive studies may clarify the boundaries within the Facelinidae subgroups.

TECTIPLEURA Schrödl *et al.*, 2011
EUOPISTHOBRANCHIA Jörger *et al.*, 2010
UMBRACULOIDEA Dall, 1889 (1827)
Family UMBRACULIDAE Dall, 1889
Genus *Umbraculum* Schumacher, 1817
Umbraculum umbraculum (Lightfoot, 1786)
(Figure 1F)

MATERIAL EXAMINED
One specimen photographed alive, material not collected.

REMARKS
Another species considered to have a wide geographical distribution in the Atlantic, Indo-Pacific and also eastern Pacific waters (Uribe *et al.*, 2013). Many names were synonymized to *Umbraculum umbraculum*, and at least some may represent valid names of more restricted distributed, cryptic, species. This species was first recorded from Ascension under the name *Umbraculum mediterraneum* (Lamarck, 1819) based on a single shell (Rosewater, 1975: p. 26). During the recent survey, a living specimen was photographed (Figure 1F) and several more were observed by the second author, confirming the occurrence of the species in the island.

ANASPIDEA Fischer, 1883
Family APLYSIIDAE Lamarck, 1809
Genus *Dolabrifera* Gray, 1847
Dolabrifera dolabrifera (Rang, 1828)
(Figure 3C)

MATERIAL EXAMINED
Two specimens, 6 and 10 mm long, preserved (under rocks in 12 m depth in Northeast Bay) (ZSM Mol 20130112), collector not recorded, 6 September 2012. One specimen, 13 mm long, preserved (under rocks on 10 m depth, English Bay) (ZSM Mol 20130106), collector not recorded, 6 September 2012.

REMARKS
This species is distributed circumglobally in tropical and subtropical waters (Rudman, 2003). However, as commented for *Micromelo undatus* and *Umbraculum umbraculum*, this wide distribution should be investigated with more detailed, comparative morphological and molecular studies. This is the first record of *D. dolabrifera* from Ascension Island.

Genus *Aplysia* Linnaeus, 1767
Aplysia parvula Guilding in Mörch, 1863

MATERIAL EXAMINED
Two specimens, 8 and 10 mm long, preserved (under rocks) (ZSM Mol 20130115), collector not recorded, September 2012.

REMARKS
This is the first record of *Aplysia parvula* from Ascension and the second record of an *Aplysia* species, after the record of *A. dactylomela* Rang, 1828 from the island by Rosewater (1975). Unfortunately, no photographs of living specimens of *A. parvula* were taken. *Aplysia parvula* is distributed in tropical to warm temperate waters worldwide, but preliminary molecular data point to the existence of a complex of species (V. Padula, unpublished data); this may represent a similar case to *A. dactylomela*, for which cryptic species were detected through molecular analysis (Alexander & Valdés, 2013).

PANPULMONATA Jörger *et al.*, 2010
SACOGLOSSA Ihering, 1876
Family CALIPHYLLIDAE Tiberi, 1881
Genus *Caliphylla* A. Costa, 1867
Caliphylla mediterranea A. Costa, 1867
(Figure 3D)

MATERIAL EXAMINED
Two specimens, 4 and 5 mm long, preserved (on the alga *Bryopsis plumosa* , 8 m depth in English Bay, at night) (ZSM Mol 20130111), coll. P. Wirtz, 8 September 2012.

REMARKS
Originally described from the Mediterranean Sea, *Caliphylla mediterranea* was later recorded from different localities in the Caribbean Sea, Brazil and Senegal (Gascoine, 1979; Padula *et al.*, 2012). It is here recorded from Ascension Island for the first time. It is unclear if the material of all these localities really belongs to the same species or represents a complex of cryptic species. Amphiatlantic distribution was supported for some, and rejected for other sacoglossan species in a recent study by Carmona *et al.* (2011).

DISCUSSION

The addition of seven new records, including two new species, almost doubles the number of 'opisthobranch' species known from Ascension Island (Rosewater, 1975). A list of all heterobranch sea slugs recorded from Ascension is given in Table 1. Due to Ascension's geographical position and isolation, a recurrent question is the origin of its shallow water fauna and flora and the degree of endemism (Price & John, 1980). Among the 10 heterobranch species studied herein, four are considered circumglobal (*M. undatus*, *D. dolabrifera*, *U. umbraculum* and *A. parvula*) and one is recorded from the eastern Pacific and both sides of the Atlantic (*P. areolatus*). Such a range of distribution would not be naturally expected, as the maintenance of genetic structure between different oceans is highly incompatible to the biology of opisthobranch species with mostly benthic life (Goddard, 2004). Recent studies on sea slug species with similar wide geographical distribution revealed the existence of cryptic species. '*Navanax aenigmaticus*', for example, is not distributed in the eastern Pacific, and on both sides of the Atlantic as previously thought, but indeed is a complex of three cryptic species, each restricted to one of these geographical regions (Ornelas-Gatdula *et al.*, 2012). Hawaian specimens of '*Aplysia dactylomela*' are not conspecific to Atlantic and Mediterranean ones (Alexander & Valdés, 2013; Valdés *et al.*, 2013). The existence of many more such cases is likely (Uribe *et al.*, 2013).

It is becoming clear that the traditional sea slug taxonomy, based on a reduced number of characters, does not allow secure delimitation of similar or even morphologically identical species (e.g. Krug *et al.*, 2013), but additionally requires sound molecular approaches (see Jörger *et al.*, 2012, Jörger & Schrödl, 2013). For the circumglobal, widespread species considered in the present work, the identification provided here is tentative until comprehensive and integrative studies elucidate the real number of species involved and the correct names to be applied.

Briggs (1974, 1995) proposed that Ascension and St Helena together constitute a separate biogeographical province in the Atlantic. Data available on more studied groups, such as reef fish, which due to their life habits and biology can be compared to some benthic invertebrates, corroborate this idea (Bullock, 1980; Floeter *et al.*, 2008). At the same time, it is known that Ascension and St Helena shallow water marine fauna receives strong influence from both the western and eastern Atlantic, including the occurrence of amphiatlantic species (Floeter *et al.*, 2008). In general, the influence of the western Atlantic seems to be stronger (Manning & Chace Jr, 1990), but see Floeter *et al.* (2008: p. 38). Briggs & Bowen (2012) compiled biogeographical data and reported the open-water expanse of the mid-Atlantic, that is, the mass of water separating the western Atlantic and the eastern Atlantic, as a soft barrier for dispersal. The almost absent data on heterobranch sea slugs from the east coast of Africa limits the discussion in our case. From one side, the discovery of two new species (*F. atlantica* and *P. mimica*), unknown from elsewhere, reinforces the biogeographical province status of Ascension (Briggs, 1974, 1995, Floeter *et al.*, 2008). On the other hand, the occurrence of the nudibranch *Platydoris angustipes*, currently only known from the tropical western Atlantic and from Ascension Island, corroborates the marine faunal affinity between these two regions.

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3. RESULTS

<u>Chapter 5</u>

Brenzinger B, **Padula V**, Schrödl M (2013) Insemination by a kiss? Interactive 3D microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Gastropoda: Euopisthobranchia: Philinoglossidae). *Organisms, Diversity & Evolution* **13**: 33-54.

ORIGINAL ARTICLE

Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Gastropoda: Euopisthobranchia: Philinoglossidae)

Bastian Brenzinger · Vinicius Padula · Michael Schrödl

Abstract Increasing molecular evidence suggests that the phylogeny of euthyneuran gastropods differs greatly from centenary textbook concepts. The presence, homology and evolution of characters in major subgroups thus need to be reinvestigated. Traditionally basal opisthobranch Cephalaspidea ("head-shield snails and slugs") were pruned to a new taxon concept, with benthic euopisthobranch and tentacle-bearing cephalaspidean lineages basal to burrowing, head-shield bearing philinoidean species. Among the latter, mesopsammic "microslug" lineages evolved at least twice. Herein we explore in 3D microanatomical detail the putatively basal philinoglossan *Pluscula cuica* (Marcus, Boletim da Faculdade de Filosofia, Ciências e Letras. Universidade de São Paulo 164:165–203, 1953a) from its type locality in Brazil. The species possesses several "accessory" ganglia and a reduced posterior mantle cavity that retains some putative shell-building tissue and an osphradium. The hermaphroditic, monaulic genital system opens in a posterior position; it retains a bursa copulatrix but lacks a distinct receptaculum seminis. Autosperm is transferred to the cephalic copulatory organ via an external sperm groove, not through the hemocoel, as suggested in the original description. The penis opens through the oral tube, sperm is transferred by a "kiss". A conspicuous yellow gland is discussed as a modified Blochmann's gland. Retaining several putative symplesiomorphies with philinoids, *Pluscula* is discussed as the most basal offshoot in meiofaunal Philinoglossidae. However, the supposed "primitiveness" of the fused rather than separate cerebropleural ganglia and the triganglionate rather than pentaganglionate visceral nerve cord was based on misobservations. Higher categories such as Philinoglossacea for Philinoglossidae, and a separate family Plusculidae for *P. cuica* are no longer warranted. Inner cephalaspidean relationships and a scenario of more or less successive philinoglossid adaptation to meiofaunal environments should be investigated by molecular studies with more comprehensive taxon sampling.

Keywords Mollusca · Opisthobranch · Meiofauna · Interstitial · Adaptation · Phylogeny

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Introduction

Gastropoda are renowned for their morphological, and therefore ecological, diversity (e.g., Beesley et al. 1998). In recent decades, phylogenetic studies have rapidly increased our understanding of their evolution. However, accumulating molecular evidence suggests that the topology of Heterobranchia — covering roughly half of gastropod diversity — differs greatly from traditional textbook concepts. The long held split of Euthyneura into monophyletic Opisthobranchia and Pulmonata has been challenged (e.g., Haszprunar 1985; Dayrat and Tillier 2002; Klussmann-Kolb et al. 2008; Dinapoli and Klussmann-Kolb 2010) and a "new euthyneuran tree" has emerged (Jörger

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et al. 2010a; Schrödl et al. 2011a, b; Göbbeler and Klussmann-Kolb 2011), the backbone topology of which has been confirmed in phylogenomic approaches (Kocot et al. 2011; Smith et al. 2011). In the light of radically changing concepts and classifications, morphological characters, taxa and traits need to be reinvestigated (Schrödl et al. 2011a).

Among the most aberrant and problematic heterobranchs are several lineages of minute slugs that are specialized members of the meiofauna. Living in the marine interstitial or mesopsammon, i.e., the interstices between sand grains in well oxygenated sands (Swedmark 1964, 1968), all these taxa — most acochlidians, rhodopemorphs, some Cephalaspidea, Sacoglossa and Nudibranchia (Arnaud et al. 1986)—exhibit characteristic morphologies. Convergently evolved characters are small sizes, vermiform bodies, losses of body appendages, eyes and pigmentation, development of adhesive abilities, spicules and additional ganglia, and unusual reproductive traits such as the production of spermatophores, hypodermal insemination, production of only few eggs, and loss of a free-floating larval stage (Swedmark 1968, 1971; Salvini-Plawen 1973; Schrödl and Neusser 2010; Neusser et al. 2011a; Schrödl et al. 2011a). Similar features and tendencies are also found in other groups of metazoans that inhabit the same habitat (Swedmark 1964; Higgins and Thiel 1988; Rundell and Leander 2010). In addition to showing reductions and convergent innovations, the reduced adult size common to these taxa is suggestive for progenetic processes (e.g., Hanken and Wake 1993). Retaining simple juvenile features means losing diagnostic apomorphies of higher clades and gaining pseudoarchaic ones; this may lead to entirely wrong classificatory conclusions (Martynov et al. 2011; Martynov and Schrödl 2011). Furthermore, minute specimen sizes have historically hampered both collecting efforts and structural analyses. Incongruities from previous descriptions were detected and corrected during 3D microanatomical reanalyses of meiofaunal sacoglossans (Rückert et al. 2008) and acochlidians (e.g., Neusser et al. 2006, 2009a; Jörger et al. 2008, 2010b; Eder et al. 2011) that were originally examined using paraffin-based histology. Interstitial cephalaspideans have not yet been analyzed in such depth.

The Cephalaspidea or "bubble-shells" were long thought to be the most basal and conservative major opisthobranch clade, including several distinct taxa characterized by the name-giving head-shield, an organ used for infaunal digging (Gosliner 1994; Mikkelsen 1996; Burn and Thompson 1998). However, the inclusiveness of the taxon concept has decreased over time. Acteonoidea and Ringiculoidea were already excluded from Cephalaspidea on morphological grounds (Haszprunar 1985; Mikkelsen 1996, 2002); the former were placed at the base or outside Euthyneura by multi-locus analyses (Göbbeler and Klussmann-Kolb 2010, 2011; Dinapoli and Klussmann-Kolb 2010; Jörger et al. 2010a; Schrödl et al. 2011a, b). The previously disputed

cephalaspidean *Cylindrobulla* (Jensen 1996; Mikkelsen 1996, 1998) was confirmed as a "bubble-shelled" sacoglossan panpulmonate by molecular analyses (Händeler and Wägele 2007; Maeda et al. 2010; Neusser et al. 2011b). Finally, Malaquias et al. (2009) removed the small-sized benthic Runcinacea from Cephalaspidea; this has been confirmed by molecular studies using larger outgroup sets (Jörger et al. 2010b; Göbbeler and Klussmann-Kolb 2011). With the remaining Cephalaspidea now appearing as a non-basal taxon within so-called Euopisthobranchia (Jörger et al. 2010a), head-shield bearing lineages are scattered over the euthyneuran tree. This reclassification has important implications for the understanding of euthyneuran evolution. For example, euthyneuran head tentacles and head shields show essentially similar cerebral innervation patterns (Huber 1993; Faller et al. 2008; Staubach et al. 2008; Jörger et al. 2010b) and thus may simply transform according to habitats and life styles.

Within Cephalaspidea, morphology-based classifications are heterogeneous and authors claimed at least four 'super-familial' ranks. The most basal Cephalaspidea in all available multi-locus studies were the little-known Diaphanoidea (e.g., Malaquias et al. 2009; Jörger et al. 2010a; Göbbeler and Klussmann-Kolb 2011). Intriguingly, this paraphyletic group (Göbbeler and Klussmann-Kolb 2011) contains tentacle-bearing members such as benthic *Colpodaspis* and infaunal *Toledonia* (Brown 1979; Golding 2010) suggesting that there is no simple ecological rule. Therefore, one might suggest that diaphanoidean tentacles may be phylogenetic remainders of a benthic euopisthobranch ancestor, while higher cephalaspideans have evolved their eponymous head-shields de novo. Stable inner cephalaspidean topologies and detailed micro-anatomical data to test these hypotheses are not yet available. Albeit with varying topologies, members of at least four families of the carnivorous Philinoidea commonly cluster close together: *Scaphander* (Scaphandridae), *Philine* (Philinidae), Aglajidae and Gastropteridae (Malaquias et al. 2009; Göbbeler and Klussmann-Kolb 2011). These philinoid families contain slender carnivores with a reduced or internalized shell (save *Scaphander*) and a rearward displaced mantle cavity (Burn and Thompson 1998). Mesopsammic, at least externally shell-less philinoideans have evolved independently at least twice (Arnaud et al. 1986; Malaquias et al. 2009; Jörger et al. 2010a): within the burrowing Philinidae (*Philine exigua* Challis, 1969a and juveniles of other species), and with the entirely mesopsammic 'Philinoglossacea' Thiele, 1931 of still unknown affinities.

The philinoglossans are a small group containing four genera and seven described species (four of which belong to *Philinoglossa* Hertling, 1932). These miniaturized slugs (body length rarely exceeds 4 mm) show a ribbon-shaped body with posteriorly overhanging dorsum, lack a distinguishable head-shield (except for the Mediterranean *Abavopsis latosoleata* Salvini-Plawen, 1973), a gill, and have at best a vestigial shell.

These multiple reductions have significantly hampered phylogenetic studies based on morphological data: Wägele and Klussmann-Kolb (2005) recovered philinoglossans within a group containing the meiofaunal members from several traditional heterobranch 'orders'. Molecular studies have shown to be better suited to solve similar tasks (e.g., Malaquias et al. 2009; Jörger et al. 2010a) but so far only a few have included philinoglossans in their sampling. Accordingly, their phylogenetic position within philinoid Cephalaspidea is not known: Vonnemann et al. (2005) recovered *Philinoglossa praelongata* Salvini-Plawen, 1973 basal but inside a polytomy. Both Malaquias et al. (2009) and Göbbeler and Klussmann-Kolb (2011) identify a clade of *Philinoglossa* and Gastropteridae as sister to Aglajidae plus Philinidae, with Scaphandridae basal. Jörger et al. (2010a) recovered *Scaphander* as sister to *Philinoglossa*, but without covering the aforementioned families. So far, monophyly of 'Philinoglossacea' has never been tested by including more than single representatives into molecular analyses. Not much is known about the biology of the group.

The monotypic genus *Pluscula* is represented by the Brazilian *Pluscula cuica* Marcus, 1953a, the only philinoglossan species described from the Americas. It is potentially the most basal of philinoglossans, since it is described with characters that appear to be plesiomorphic and are not found in the other genera (Marcus 1953a). These characters are a thin internalized shell, the genital opening in a posterior position, still separate cerebral and pleural ganglia, and five distinguishable ganglia on the visceral nerve cord. On the other hand, the mode of autosperm transfer is suggested to be unique and peculiar: Marcus (1953a) observed numerous spermatozoa in the body cavity and concluded that autosperm move from the gonad directly to the copulatory organ—through the hemocoel, instead of being transported along the external ciliated groove running along the right body side, as in most other cephalaspideans. Due to these peculiarities, some authors place *Pluscula cuica* in a family of its own (Plusculidae: Marcus 1959; Franc 1968; Bouchet and Rocroi 2005) or subfamily (Plusculinae: Salvini-Plawen 1973). Therefore, *Pluscula cuica* might be a key organism for the understanding of philinoglossan evolution and the internal phylogeny of philinoid groups, and interesting for its peculiar reproductive mode.

Within a framework of comparative morphological and evolutionary studies on mesopsammic heterobranchs, we analyzed the entire microanatomy of *Pluscula cuica* using computer-based 3D reconstruction from semi-thin histological sections. Our aims were to (1) check, correct, and supplement the original description; (2) elucidate the structure and function of the reproductive system, in particular with regard to the potentially highly peculiar modes of autosperm transport and transfer; and (3) evaluate potentially ancestral features in a phylogenetic context, reconsidering the familial status of the species, and the relationships of philinoglossans to other cephalaspideans.

Materials and methods

Specimens of *Pluscula cuica* were extracted from bulk samples of coarse sand taken from the uppermost subtidal at low tide at Ilhabela, São Paulo, Brazil (type locality) in 2005 following the method described by Schrödl (2006). Specimens were relaxed in isotonic magnesium chloride solution, fixed in ethanol (75 % or 96 %) or, for histology, in 4 % glutaraldehyde (in 0.2 M cacodylate buffer, 0.1 M sodium chloride, 0.35 M sucrose buffered at pH 7.2). The latter specimens were further postfixed with 1 % osmium tetroxide in 0.2 M cacodylate buffer/0.3 M sodium chloride, then dehydrated over a graded acetone series and embedded in Spurr's epoxy resin (Spurr 1969). Specimens are stored at the Bavarian State Collection of Zoology (ZSM), Department Mollusca, Munich, Germany, and in the malacological collection of Museu de Zoologia da Universidade de São Paulo (MZSP, vouchers 104098–104100), Brazil (C. M. Cunha, personal communication).

For 3D reconstruction, three specimens in epoxy blocks were trimmed and serially sectioned at 1.5 µm using either Ralph glass knives (specimens ZSM Mol-20070316, 20070323) or a HistoJumbo diamond knife (specimen ZSM Mol-20070317) (Diatome, Biel, Switzerland) with contact cement at the lower cutting edge, following the method described by Ruthensteiner (2008). Ribbons of sections were collected on microscope slides, stained with methylene blue/azure-II (Richardson et al. 1960) and sealed with araldite resin. Sections of the complete diamond-sectioned specimen—a moderately contracted adult specimen of approximately 1.7 mm length—and, separately, its central nervous system were photographed with a ProgRes C3 ccd camera (Jenoptik, Jena, Germany) mounted on a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Photographs were stack processed (resized, changed to greyscale, unsharp masked) in Adobe Photoshop (Adobe Systems, Mountain View, CA) and imported into Amira 5.2 software (Visage Imaging, Berlin, Germany) with a resolution of 1,024×768 or 2,080×1542 pixels, respectively. After alignment of the photographs, organ systems were labeled manually onto the sections. Rendered 3D models of the organ systems were created for the complete specimen (based on 575 photographs, every second section was used). Details of the specimen's nervous system were analyzed in a separate aligned stack (256 photos, every section used), but labeled in the complete body's model. Anatomical features were compared among all three specimens (one juvenile, one functionally male, the other adult).

Further two specimens fixed in 75 % ethanol (lot: ZSM Mol-20070835) were photographed through a Leica dissection microscope and macerated in KOH solution for analysis of shell remnants and the radula. Radulae were viewed through above mentioned light microscope for counting of tooth rows and detection of denticulate tooth margins.
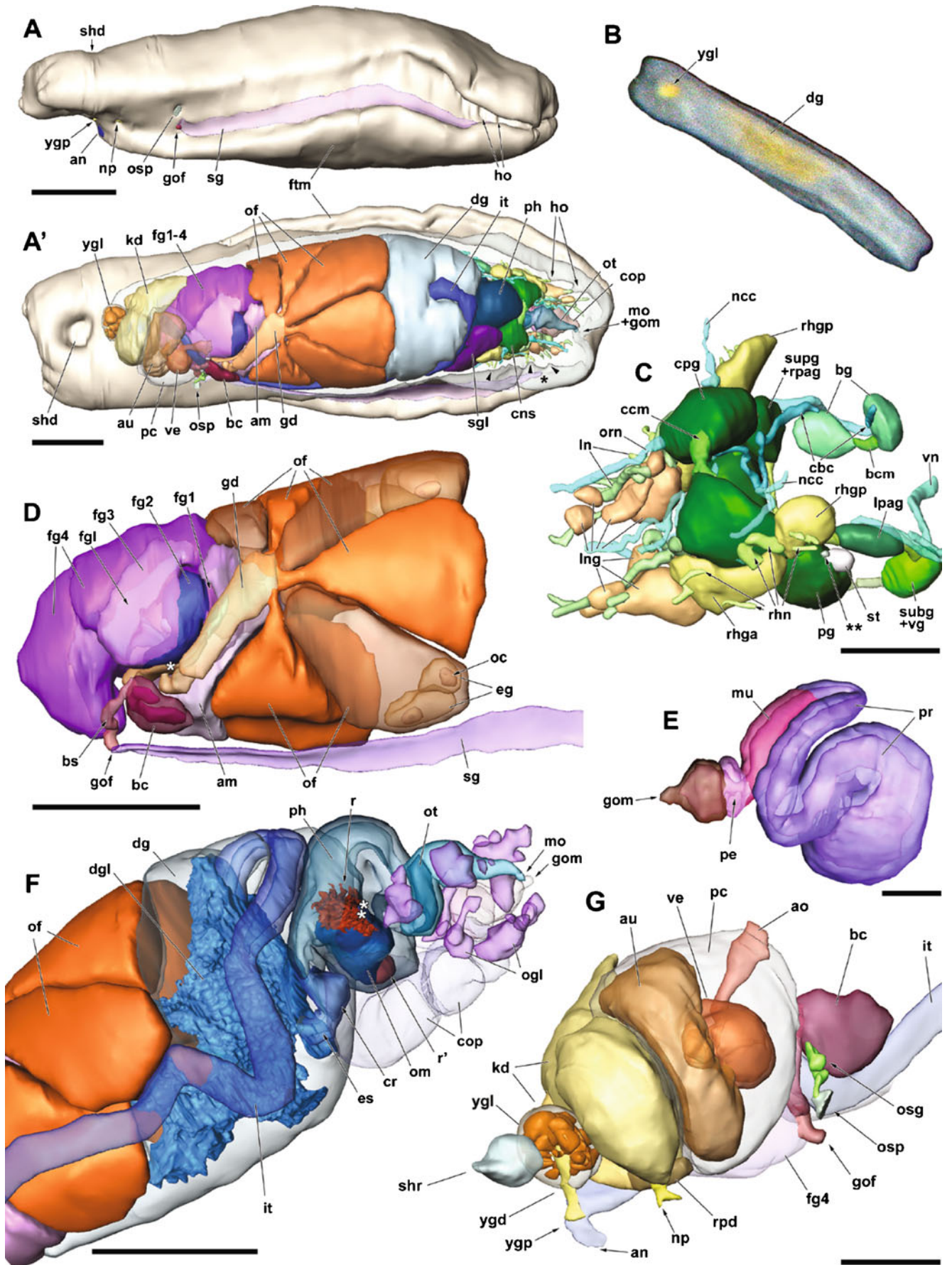
Fig. 1 **a–g** Three-dimensional reconstructions of *Pluscula cuica* microanatomy. **a** External aspect of body showing body openings, right view. **a'** Dorsal view of body with the dorsum above body cavity and head shown transparent, showing inner organ systems, *arrowheads* short nerves innervating Hancock's organs, *asterisk* anterior end of seminal groove. **b** Live specimen, ca. 2 mm total length, dorsal view. **c** Anterior left view of the central nervous system, pedal nerves omitted, *double asterisk*: large cell next to statocyst, **d** Posterior part of reproductive system, dorsolateral right view, *white asterisk* branching point of gonoduct to female glands and ampulla. **e** Copulatory apparatus, ventral view, anterior towards left. **f** Oblique right view of digestive system, salivary glands omitted, *double white asterisks* positions of salivary duct openings and small glandular field inside pharyngeal lumen. **g** Oblique dorsolateral right view of pericardial complex and surrounding organs. *am* ampulla, *an* anus, *ao* aorta, *au* auricle, *bc* bursa copulatrix, *bcm* buccal commissure, *bg* buccal ganglion, *bs* bursa stalk, *cbc* cerebro-buccal commissure, *ccm* cerebral commissure, *cns* central nervous system, *cpg* cerebropleural ganglion, *cop* copulatory apparatus, *cr* putative crop, *dg* digestive gland, *dgl* lumen of digestive gland, *eg* egg, *es* esophagus, *fg1–fg4* nidamental glands (proximal to distal), *fgl* lumen of nidamental glands, *gd* gonoduct, *gof* female genital opening, *gom* male genital opening, *ho* Hancock's organs, *it* intestine, *kd* kidney, *ln* labiotentacular nerve, *lng* accessory labiotentacular ganglia, *lpag* left parietal ganglion, *mo* mouth opening, *mu* muscular tube, *ncc* nervus clypei-capitis, *np* nephropore, *oc* oocyte, *of* ovarial follicles, *ogl* oral glands, *om* odontophore musculature, *orn* oral nerve, *osg* osphradial ganglion, *osp* osphradium, *ot* oral tube, *pc* pericardium, *pe* penis, *pg* pedal ganglion, *ph* pharynx, *pr* prostate, *r* distal part of radula, *r'* origin of radula, *rhga/rhgp* anterior/posterior accessory rhinophoral ganglion, *rhn* rhinophoral nerve, *rpd* renopericardial duct, *sg* seminal groove, *sgl* salivary gland, *shd* shell dimple, *shr* shell remnant, *st* statocyst, *subg+vg* combined subintestinal and visceral ganglion, *supg+rpag* combined supraintestinal and right parietal ganglion, *ve* ventricle, *vn* visceral nerve, *ygd* duct of yellow gland, *ygl* yellow gland, *ygp* opening of yellow gland. *Bars* **a**, **a'**, **d**, **f** 250 μm; **c**, **e**, **g** 100 μm. Interactive version of this figure is available in the supplementary online material.

The interactive model was prepared following the protocol of Ruthensteiner and Heß (2008), using Adobe Acrobat 9.0 Professional Extended software. The model can be accessed in the supplementary online interactive version of Fig. 1.

Results

Remarks on taxonomy

Euthyneura Spengel, 1881: Tectipleura Schrödl et al., 2011a: Euopisthobranchia Jörger et al., 2010a

    Cephalaspidea P. Fischer, 1883: Philinoidea Gray, 1850: Philinoglossidae Hertling, 1932 (or Plusculidae Marcus, 1959)

    Pluscula cuica Marcus, 1953a (type by monotypy)

Marcus (1959) separated monotypic Plusculidae from the Philinoglossidae Hertling, 1932 (type species *P. praelongata* Hertling, 1932) based on *P. cuica* retaining a reduced circular shell, the separation of cerebral and pleural ganglia, and the posterior position of the genital opening. Other described distinguishing features include lack of eyes, presence of five distinguishable ganglia on the visceral loop and the derived mode of autosperm transport from gonad to copulatory organ (via the hemocoel), among others. Bouchet and Rocroi (2005) used Plusculidae Franc, 1968. In contrast, Salvini-Plawen (1973) used a philinoglossid subfamily Plusculinae. Other authors included *Pluscula* and all other genera among Philinoglossidae (e.g., Arnaud et al. 1986).

While generally considered as part of the Philinoidea (e.g., Burn and Thompson 1998; Bouchet and Rocroi 2005), earlier authors commonly used the now obsolete 'order' Philinoglossacea sensu Thiele, 1931 of equal rank to Cephalaspidea (e.g., Marcus and Marcus 1954; Salvini-Plawen 1973). For practical reasons, we use the term 'philinoglossan' to address *Pluscula cuica* and the three other philinoglossid genera.

General anatomy and histology

Living specimens of *Pluscula cuica* are white, with externally visible yellowish digestive gland and the conspicuous 'yellow' gland in the caudal part (Fig. 1b). The body is approximately rectangular in dorsal aspect, and about 3.5 to 4.5 times longer than wide (ca. 1.7 mm × 500 μm in the reconstructed specimen), with a smooth epidermis. The dorsal side is slightly convex; head shield and notum are fused without a detectable groove. The head end is concave with rounded corners. The overhanging posterior end of the notum has a dimple on top under which where remnants of the shell-forming tissue are located; the depression appears to be more pronounced in fixed specimens. Slightly more anterior, the conspicuous spherical yellow gland may be visible, if filled (Fig. 1a',b). Four body openings that are usually found inside the mantle cavity are located underneath the right side of the posteriorly overhanging notum (Fig. 1a). Notum and foot are separated by wide longitudinal grooves along the circumference of the body; the grooves are widest on the sides of the head, thinnest along the anterior face of the body, left and right to where the mouth is situated. The foot is only slightly indented anteriorly, it is wider than the notum in the anterior half of the body; posteriorly, the foot is shorter than the notum with a slightly pointed, but not projecting end.

Notum and foot sole show a distinct margin of short motile cilia. Small intraepidermal, light pink glands can be found, especially close to the head; numerous larger pink-staining and fewer dark blue glands are located subepithelially and open to the outside via thin ducts (Fig. 4a). Within the lateral grooves, the epidermis is thinner and lacks glands and contingent ciliation except for interspersed multiciliated cells and the motile cilia of the seminal groove. Left and right of the head, the Hancock's organs are three shallow depressions with dense microvillous border (Fig. 1a,a'; 4d).

Below the epidermis there is loose connective tissue (formed by round cells that contain an unstained vacuole) that is intersected by muscle fibers, especially in the foot.

Instead of the previously described shell, the decalcified examined specimens show only a dense batch of blue-staining, irregularly sorted fibrous material located within the connective tissue of the overhanging notum end, just below the dorsal depression (Fig. 5g). This circular shell organ/vestige (80 μm diameter, 55 μm thick; Fig. 1g) lacks any trace of a dissolved shell.

The main body cavity is round in cross-section along most of the body's length and separated from the outer connective tissue by a strong layer of mostly longitudinal muscle fibers. All major organ systems reconstructed herein are situated within this body cavity (Fig. 1a). A diaphragm is not detectable.

In the most posterior end of the body cavity lies a conspicuous gland which is visible in living specimens as a bright orange-yellow spot (Fig. 1a',b). The gland is roughly spherical (diameter 100 μm) and surrounded by a thin sheath of muscle fibers. It comprises large, columnar cells with a vacuole that in most cells contains remnants of a grey-staining liquid. The cells are of apparently holocrinous nature and discharge into a central epithelial duct (Fig. 5f); the duct opens to the outside just dorsal of the anus (Fig. 1g).

Digestive system

The mouth opening is located medially within the transversal groove separating notum and foot (Fig. 1a'). The oral tube is thin-walled, surrounded by irregular arrangements of pink-staining, single-celled glands (Fig. 4a). Approximately 50 μm from the outside, the copulatory organ branches from the ventral side of the tube. Following this split, the oral tube becomes wider, its inner wall with numerous longitudinal folds, indicating strong extendibility of this part (Fig. 4b,c). There are approximately ten elongate to egg-shaped, light pink-staining oral glands or various sizes situated around the oral tube (Fig. 1f, 4a); a connection to the tube's lumen is, however, detectable only in some.

The pharynx is elongate and curved (Fig. 1f). Its anterior part curves upward, is spacious and comparably thin-walled; in KOH-macerated specimens the pharynx reveals a thin cuticular covering. The posterior part of the pharynx curves downward, is more muscular and contains the odontophore in its ventral portion (Fig. 1f). There are small patches of violet-staining glandular cells to the left and right of the open radula (Fig. 4e). Inside the odontophore, thick longitudinal muscle fibers run parallel to the posterior two thirds of the still folded radula; only the anterodorsal part of the radula is spread open, underlain by paired fluid-filled lacunae. The radula itself has no distinct descending limb and lacks rhachidian teeth; there are approximately 16–20 rows of curved, pointed lateral teeth (six per row). The inner laterals are the largest and are widest at one-quarter of their height (masticatory border); the second and third laterals are smaller and grow continuously thinner towards the tip

(Fig. 4e). Neither serial sections nor light microscopic observation of the radula showed serration of the first laterals (not shown).

The salivary glands are voluminous tubes, their cells filled with comparatively few droplets of dark-blue staining secretion. In the reconstructed individual, the right salivary gland is situated ventrally and appears considerably larger; its ciliated salivary duct can be traced to the right intersection of the thin-walled and muscular walls of the pharynx (white asterisks in Fig. 1f). The left salivary gland is situated dextrodorsally and appears much smaller (Fig. 1a'). The ciliated esophagus exits the pharynx posteriorly and curves downward where it forms a spherical chamber (a vestigial crop?; Fig. 1f); esophagus and putative crop show the longitudinal folds also found in the oral tube. From there a thinner part connects to the stomach dextroventrally. A histologically distinct stomach is not detectable; the presumed stomach lumen appears to extend dorsally, towards the intestine. The digestive gland—pale yellow in living specimens, Fig. 1b—is an externally smooth sac, its outer wall is covered by a mesh of criss-crossing muscle fibers. The digestive gland's rounded anterior face fills much of the body cavity, its posterior face slopes downward (also visible in living specimens) and ends in an elongate tip at about two thirds of the body's length (Fig. 1f). The digestive lumen is outlined irregularly by an epithelium formed mainly by high columnar cells that are rounded apically (surface shown in Fig. 1f) and filled with blue-staining droplets (Fig. 4g, 5c).

The origin of the ciliated intestine is pushed into the digestive lumen in an about 70 μm long trunk-like extension at the anterodorsal side (Fig. 4g); its connection to the stomach is unclear. From there, the intestine curves to the right and runs backwards along the body side to the end of the body, where the anus is situated medially, just dorsal of the foot sole's posterior tip (Fig. 5f).

Central nervous system

The cerebral nerve ring is situated prepharyngeally and most of its ganglia adhere closely to the dorsal and lateral sides of the pharynx (Fig. 1a'). In all ganglia, neurons are situated peripherally just underneath a blue-staining fibrous layer, with central fibrous neuropil extending to the outside as nerves. Accessory ganglia can be distinguished histologically by their distinctly smaller neurons and less obvious separation into cortex and neuropil (Fig. 4b–d).

The paired cerebropleural ganglia are the largest ganglia and are connected by the thick cerebral commissure; each ganglion is hemispherical anteriorly and oblong posteriorly. The cerebropedal and pleuropedal connectives connect each cerebropleural ganglion to the pedal ganglia. The connectives to the ganglia on the visceral loop (pleuroparietal c.) are short (left side) and very short (right side). The

cerebrobuccal connectives are long and slightly undulated; they emerge from the medioventral side of each cerebropleural ganglion and run along the sides of the pharynx. Only the right cerebro-buccal connective could be traced along its entire length.

From each cerebropleural ganglion, four nerves emerge and run laterally and frontally. The anterior and median oral nerve is of medium thickness and appears to innervate the oral tube and mouth opening; on the left side, this nerve shows a distal bifurcation. Slightly more laterally, the very thick labiotentacular nerve emerges; this nerve shows two branches that are equipped with several accessory ganglia: the lateral branch innervates a large ganglion (70×50 μm), the median branch shows along its length four smaller ganglia (25–40 μm) that are closer to the digestive tract. On the left side, the first of the small ganglia and the large ganglion are partially fused. The large ganglion emits several short nerves innervating the most anterior epidermal pit in position of the Hancock's organ, while the smaller ganglia show nerves running medially, towards the oral tube and mouth opening.

Two further nerves emerge from the sides of each cerebropleural ganglion. One is thin and extends dorsolaterally (headshield nerve; Fig. 1c). The rhinophoral nerve is very thick (diameter 20 μm) and emerges laterally; it shows a rather wide connection to the cerebropleural ganglion with possibly two separate roots in the cerebro-pleural ganglion. The rhinophoral nerve splits close to its base, each part supplying two large accessory ganglia: the anterior one is elongate and about 100 μm long, the posterior one is situated more posterodorsal and oval (70×50 μm). Again, each ganglion innervates sensory cells in pits of the Hancock's organs via at least two to three short nerves (Fig. 4d). A fifth cerebral nerve, thin and running to the oral tube, was detected only on the left side, emerging anterior of the left cerebrobuccal connective. *Pluscula cuica* lacks eyes.

The paired buccal ganglia are of medium diameter and situated at the posterior side of the pharynx just below the origin of the esophagus, under which the buccal commissure passes. Buccal nerves could not be detected.

The paired pedal ganglia are almost spherical and connected by the long pedal commissure. Several nerves of different diameter originate from each ganglion, in general running to the body sides and into the foot. One anterior-running nerve emerges just next to the cerebropedal connective, two nerves emerge close by on the anteroventral face of the pedal ganglion and run anteriorly, and a very thick posterior nerve exits from the posteroventral side. A further posterior-running nerve was found only on the left side, while a dorsolateral nerve emerging just anterior to the statocyst was detected only on the right.

The spherical statocysts are located on the posterodorsal side of each pedal ganglion; each statocyst is of approximately 30 μm diameter and contains a single statolith (Fig. 4f). The static nerve could not be detected. Just anterodorsally to the statocysts of both sides there is a conspicuous 'blister'-like cell containing a large unstained vesicle or vacuole (Figs. 1c, 4f).

There are three medium-sized ganglia on the euthyneurous visceral loop; two are close together on the left side (1, the left parietal and 2, the combined subintestinal and visceral ganglion; terminology after Haszprunar 1985), the third (combined supraintestinal and right parietal ganglion) being situated just behind the right cerebropleural ganglion. Ganglia two and three are connected by a very long connective passing below the pharynx close to the pedal commissure. The left parietal ganglion is elongate and shows a single nerve curving to the left body side. Ganglion number two (medium-sized, rounded) shows two nerves: the left one thin, the right one (visceral nerve) very thick. Both nerves run posterior inside the body cavity. Ganglion number three (medium-sized) shows another very thick nerve running posterior along the right side of the body cavity.

An additional ganglion, consisting of two to three small lobes, can be found between the female genital opening and the sac of the bursa copulatrix (Fig. 1g). The connection to the central nervous system (CNS) could not be clarified, but there is a short nerve running to a small ciliated pit located inside the right lateral groove just dorsal to the genital opening. This pit consists of higher cells than the surrounding epidermis and might represent a small osphradium (Fig. 5e); we therefore regard the associated ganglion to be an osphradial ganglion.

Pericardial complex

The pericardial complex comprises the main parts of the circulatory and the excretory systems and fills the posterior end of the body cavity.

The circulatory system consists of the thin-walled pericardium, broad auricle and oval ventricle and is located at the posterior right of the body cavity (Fig. 1a'). The auricle is almost as wide as the posterior end of the pericardium and curves around the more anterior ventricle (Fig. 1g). The proximal end of the ventricle is equally thin-walled but shows a transversal, valve-like septum separating left and right (Fig. 5d); the ventricle's distal tip points marginally to the left and has a slightly thicker, muscular wall from which the aorta emerges. The aorta exits the pericardium at its anterior tip; it runs along the upper right of the body wall, parallel to the intestine. Right of the pharynx it splits into two thin-walled hemolymph vessels (Fig. 4c,f); one turns left, runs below the pedal commissure and then anteriorly, the other passes the CNS on the right and terminates close to the oral tube (not shown).

The horseshoe-shaped kidney broadly touches the posterior wall of the pericardium and expands to the left; it is characterized by the typical vacuolate, unstained epithelium. The ciliated renopericardial duct exits from the posterior right end of the pericardium and curves to the left, leading into the thinner limb of the kidney. This runs into the larger part of the kidney at the left body side, which then curves to the front and right again. A very short and thin nephroduct connects to the renal pore located inside the longitudinal groove just right of the foot's tip (Fig. 1g).

Reproductive system

Pluscula cuica is a monaulic hermaphrodite with follicular gonad, posterior right genital opening, ciliated sperm groove on the right body side and copulatory organ opening through the mouth. The posterior part of the reproductive system fills about half of the body cavity.

In the reconstructed specimen, the gonad (ovotestis) consists of six thin-walled, cone-shaped follicles that radiate from a common mid-dorsal position in the gonoduct (Fig. 1a',d). The follicles are widest at the base where they touch the lateral and ventral body wall or the sloping posterior part of the digestive gland (Fig. 1f). Spermatozoa with screw-shaped head fill most of the follicles' volume (Fig. 5c) and are arranged around large nursing cells. Except for the most dorsal, each follicle also contains two to three oocytes in various stages of development (bright nucleus and blue-staining nucleolus without surrounding yolk, or with various amounts of blue-staining yolk droplets) in its ventral portion. Other cell types—gamete precursors or types of nursing cells—are loosely arranged around the periphery of the follicles.

All follicles discharge via short stalks into the dorsally situated gonoduct, a ciliated tube that runs posteriorly, then curves downward. A short stalk (white asterisk in Fig. 1d) leads downward and connects via a small pore to the very large ampulla—a thin-walled sac filled densely with spermatozoa and extending anterior between the gonad's follicles. Unusually for this organ, the walls of the ampulla are irregularly covered with large cells filled with up to ten very large blue-staining droplets (lipids?) (Fig. 5a,b). The postampullary gonoduct curves to the left, forming the nidamental gland mass with a thick and strongly glandular wall and irregularly shaped ciliated lumen. The entire gland mass consists of three, possibly four histologically different parts, three of which form the more convoluted but thinner part running to the left. The first gland (albumen gland) is a short tube characterized by rounded, light blue/pinkish staining cells with gaps between them (Fig. 5b); the second gland (membrane gland) is equally short and has more columnar cells filled with dark blue-staining small droplets (Fig. 5a); the third gland (mucus gland, proximal limb) is an elongate tube and shows columnar, pink-staining cells. Between glands one and two, the

gonoduct wall forms a thin-walled pouch expanding dorsally (another connection could not be found); this pouch is filled densely with spermatozoa (Fig. 5b). It is not clear whether these are auto- or allosperm. The third nidamental gland turns downward. From the turning point on, a uniform part of the gland mass (mucus gland, distal limb) crosses the entire body cavity in a wide curve; its wall resembles that of gland three in histology but is much thicker (cells are at least twice as high and stain slightly darker pink) (Fig. 5a,b). Close to the right body wall, the distal gonoduct becomes non-glandular again for a short distance before opening to the outside; in this part a thin duct splits off and runs straight dorsally (Fig. 5e). Near the end of this duct a spherical pouch (bursa copulatrix) is located at the right body wall (Fig. 1d,g); the bulb is smooth on the outside and shows a more irregular inner surface, its
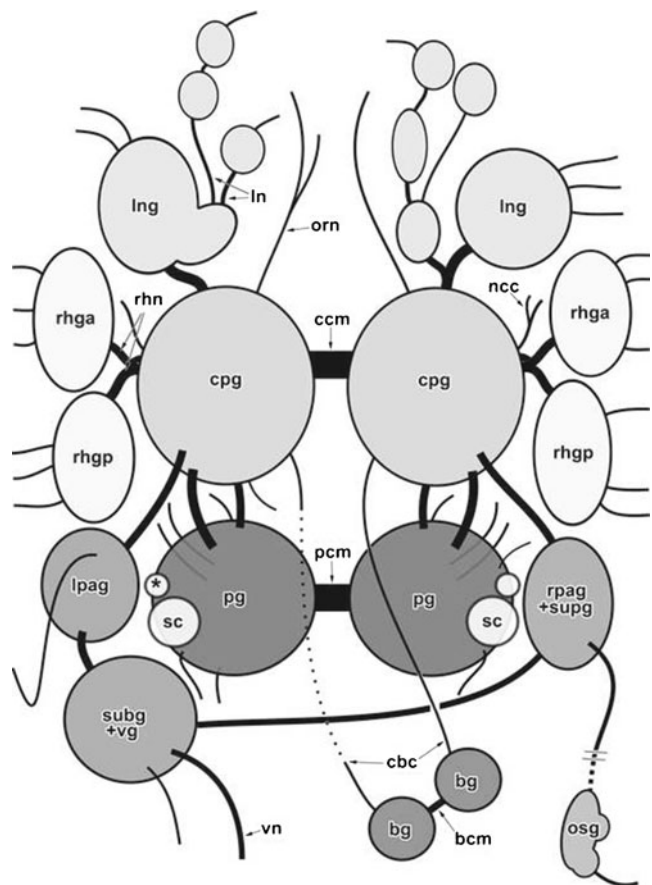
Fig. 2 Schematic dorsal view of the central nervous system (CNS) and nerves, anterior at top. Roughly to scale except for length of pleuro-parietal connectives. *bg* buccal ganglion, *bcm* buccal commissure, *cbc* cerebro-buccal connective, *ccm* cerebral commissure, *cpg* cerebropleural ganglion, *ln* labiotentacular nerve, *lng* accessory labial nerve ganglion, *lpag* left parietal ganglion, *ncc* nervus clypei-capitis, *osg* osphradial ganglion, *orn* oral nerve, *pcm* pedal commissure, *pg* pedal ganglion, *rhga* anterior accessory rhinophoral ganglion, *rhgp* posterior accessory rhinophoral ganglion, *rhn* rhinophoral nerve, *rpag+supg* combined supraintestinal and right parietal ganglion, *sc* statocyst, *subg+vg* combined subintestinal and visceral ganglion, *vn* visceral nerve, *asterisk* large 'blister' cell next to statocyst
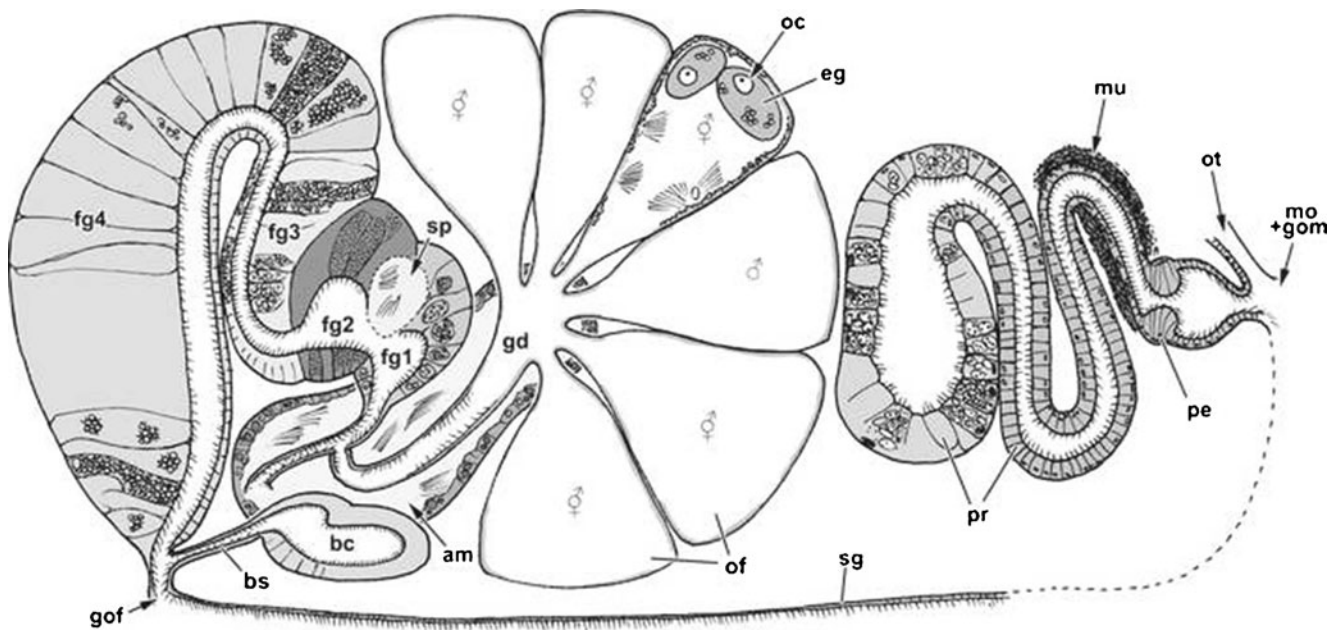
Fig. 3 Schematic dorsal view of the reproductive system, anterior at right. *am* Ampulla, *bc* bursa copulatrix, *bs* bursa stalk, *eg* egg, *fg1* albumen gland, *fg2* membrane gland, *fg3* thin portion of mucus gland, *fg4* large portion of mucus gland, *gd* gonoduct, *gof* female genital opening, *gom* male genital opening, *mo* mouth opening, *mu* muscular tube, *oc* oocyte, *of* ovarial follicles, *ot* oral tube, *pe* penis, *pr* prostate, *sp* sperm package, *sg* seminal groove

lumen is filled with a homogeneous pink-stained fluid (Fig. 5a). The genital opening is a small pore located ventrally in the right lateral groove (Fig. 1a). From the genital opening, a wide ciliated ribbon runs along the ventral portion of the right lateral groove (Figs. 1a,d,g, 5a). The ciliated strip (or sperm "groove") disappears approximately at the level of the pharynx (asterisk in Fig. 1a'), so that there appears to be no further specialized structure for sperm transport to the opening of the copulatory organ within the oral tube. In the foot margin below the end of the sperm groove, there is a group of additional glandular cells that open below the sperm groove.

The copulatory organ opens together with the mouth (Fig. 4a). It is a convoluted, blind-ending tube and extends ventrally in the body cavity as far back as the pharynx (Fig. 1a',f). It connects to the outside via a ciliated duct lined with a regular epithelium of light blue-staining cells with basal nuclei. At first the duct expands slowly before forming an almost spherical pouch, its lumen containing few spermatozoa (Figs. 1f, 2 and 3). The posterior wall of this hollow structure is considerably thicker and forms a circular rim projecting into the lumen, likely forming a penial papilla when everted to the outside (Figs. 1e, 4b). Pouch and papilla are followed by an elongate tube curving to the left; this tube shows only thin epithelial lining but is surrounded by a conspicuous mantle of thick, circular muscle fibers (Fig. 4c). The following prostate is the largest part of the copulatory organ and forms three loops before ending blindly. Its walls are thick, ciliated and glandular; the cells are

filled with unstained vacuoles and mostly apically distributed blue-staining droplets (Fig. 4c,f).

The smaller examined specimen proved to be functionally male. Its gonad consists of six follicles (two large, four smaller) that contain only spermatogenesis. The gonoduct is long, sinuous and non-glandular. There is a comparatively small ampulla with characterstic histology (blue vacuoles in epithelium). The bursa is small and empty. The copulatory organ is small but shows all elements found in the larger specimen.

Discussion

As expected, histological examination of semithin sections and 3D models generated a detailed dataset of microanatomical information with the potential to correct and/or supplement the original description of *Pluscula cuica* Marcus, 1953a. We compare these data to those available in other philinoglossans, with focus on their relationship to other cephalaspideans and in the light of new euthyneuran systematics that were established by recent molecular approaches.

External morphology and habit

Pluscula cuica can be identified by its typical philinoglossan streamlined habit without an external shell or distinct head-shield. The body is ribbon-shaped and elongate, although less than in *Philinoglossa praelongata* (see Arnaud et al. 1986). The cephalaspidean head-shield is either absent
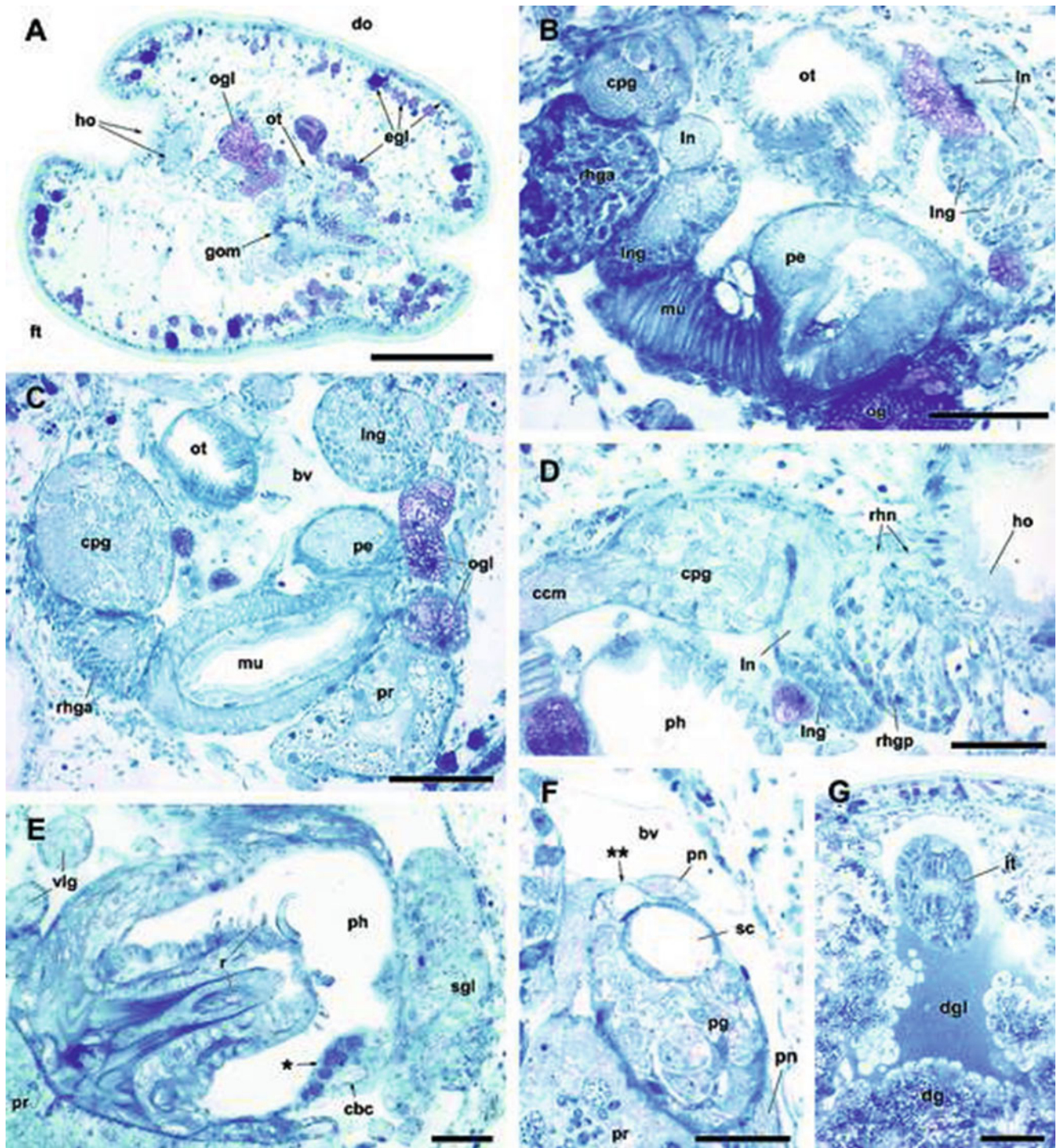
Fig. 4 a–g Semithin histological cross-sections of anterior body half. Dorsal side at *top*, in **e**: at right. **a** Level of mouth opening, showing lateral grooves. **b** Anterior part of CNS and copulatory organ. **c** Section of CNS and copulatory organ posterior to **b**. **d** Detail of right Hancock's organ and its innervation. **e** Pharynx with muscular odontophore and spread radula; *asterisk* patch of glandular cells. **f** Detail of pedal ganglion with statocyst and 'blister' cell (*double asterisk*). **g** Trunk-like anterior end of intestine inside digestive gland lumen. *bv* blood vessel, *cbc* cerebro-buccal connective, *ccm* cerebral commissure, *cpg* cerebro-pleural ganglion, *dg* digestive gland, *dgl* lumen of digestive gland, *do* dorsum, *egl* different types of epidermal glands, *ft* foot, *gom* male genital opening, *ho* Hancock's organ, *it* intestine, *ln* labiotentacular nerve, *lng* accessory labiotentacular ganglion, *mu* strong muscular lining / muscular tube of copulatory organ, *ogl* oral gland, *ot* oral tube, *pe* penis, *ph* pharynx, *pn* pedal nerves, *pr* prostate, *rhga* anterior accessory rhinophoral ganglion, *rhgp* posterior accessory rhinophoral ganglion, *rhn* rhinophoral nerve, *sc* statocyst, *sgl* salivary gland, *vlg* visceral loop ganglia (sectioned at margins). *Bars* **a** 100 µm; **b–e, g** 50 µm; **f** 25 µm

or modified into a shield confluent with the rest of the notum. We prefer the second interpretation, since the anterior part of the *Pluscula* shield is cerebrally innervated. Also, a vestigial separation of the head and body shields by a transversal groove in the first quarter of the body is present in another philinoglossid, *Abavopsis latosoleata* (Salvini-Plawen 1973, own observations). As in most other philinoglossans, the broad dorsum and foot are separated by lateral grooves that create a more or less x-shaped aspect in cross-section (an exception is *Sapha* Marcus, 1959, which is more or less round). Histological similarity of notum and foot surfaces (ciliated epithelium, epidermal glands) might be associated with the ability to crawl on either body side (observed by Hughes 1991), since all-around ciliation is present in many small-sized interstitial heterobranchs and facilitates movement between sand grains (Swedmark 1968). The foot of *Pluscula* is slightly wider than the notum and might reflect vestigial cephalaspidean parapodia. These lateral foot extensions are more pronounced in *Abavopsis,* which shows foot margins that curve upward (Salvini-Plawen 1973). This is slightly less the case in *Philinoglossa*, and *Sapha* shows only indistinct foot margins (Marcus 1959). Parapodia are a feature found in most philinoids (Burn and Thompson 1998), so the presence of a widened foot in *Abavopsis* and *Pluscula* might reflect the ancestral condition.

Pluscula shows the typical caudal overhang of the notum, underneath which the body openings are located in the body wall (see below). The caudal overhang of *Pluscula* is broad and fin-like as in *Abavopsis* and the *Philinoglossa* species; where it was observed to form a bilateral symmetric cavity if the overhang is bent downwards (Salvini-Plawen 1973). In *Sapha*, the overhang is pictured as short and pointed (Marcus 1959); an undescribed '*Philinoglossa*' from Fiji (Morse 1987) resembles this species in that aspect.

In their elongate habit and reduced shell, philinoglossans resemble most the aglajid genera *Philinopsis* or *Nakamigawaia* of which some are infaunal burrowers (Gosliner 1980). These taxa however have a fairly long head-shield (half of body length or longer) in contrast to the vestigial head-shield found in *Abavopsis*, which is rather short as in Gastropteridae (Salvini-Plawen 1973; Gosliner 1989).

Shell remnants and mantle cavity associated organs

Pluscula cuica and all other philinoglossans are externally shell-less and show a reduced mantle cavity that is roofed by the caudal overhang of the mantle. In *Pluscula*, within a short stretch of epidermis on the right body side there is the anus, the yellow gland opening, the nephropore, the genital opening and the osphradium. *Pluscula cuica* was described to possess a small internalized circular shell below a dorsal depression in the caudal end (Marcus 1953a), neither of which is present in any other philinoglossan. While not easily visible in live *Pluscula*, the dimple is quite distinct in the preserved ones examined in this study. However, no remainders of a decalcified shell in macerated specimens, or remnants of an organic matrix or empty spaces in histological sections were observed. Still, the presence of putative vestigial shell-forming tissue just underneath the dimple was confirmed herein, and this is interpreted as an ancestral feature that apparently was lost in (all?) other philinoglossans. Other features present in the putatively ancestral philinoidean mantle cavity and known only for *Pluscula* but no other philinoglossan are the osphradium (detected herein for the first time) and the genital opening associated with the mantle cavity (see the respective chapters).

The spherical yellow gland found in the caudal overhang of philinoglossans is a conspicuous histological feature and visible in many live specimens. In *Abavopsis* and *Philinoglossa* (except *P. marcusi* Challis, 1969b) it is described as an externally visible bright orange spot (Salvini-Plawen 1973, 1984), implying a strongly yellow secretion. In *Sapha*, the gland is located in the pointed tail end (Marcus 1959). Since filled glands apparently turn black by certain preserving agents, Salvini-Plawen (1984) considered them to be homologous with the 'black larval kidney' found in some other heterobranchs, implying paedomorphism (see Haszprunar 1985). These organs are in fact described to be present in larval *Philinoglossa* (Swedmark 1968), but are reduced during metamorphosis—otherwise they would be visible as conspicuous black bodies, as observable, e.g., in some post-metamorphic *Philine* (Horikoshi 1967). Alternatively, Salvini-Plawen (1973) suggested the gland to be part of an adhesive mechanism that he observed in *P. praelongata*: this species supposedly attaches to sand grains by its tail end, aided by 'glands of the epidermis and the pallial gland'. This was not observed for other philinoglossans yet but might well represent an adaptation similar to other members of the meiofauna. Some of these show localized adhesive mechanisms, e.g., rhodopemorphs that possess a caudal adhesive gland that are likely derived from glands of the foot sole (Brenzinger et al. 2011b), and thus not homologous to the gland in philinoglossans.

The nomenclature of glands located in the floor or roof of the mantle cavity in traditional opisthobranch taxa is confusing (see e.g., Wägele and Klussmann-Kolb 2005; Wägele et al. 2006 for review), therefore homologies are difficult to establish. With respect to the yellow gland of *Pluscula*, position and histology—large unstaining vacuoles in the spherical gland surrounded by muscle fibers, with epithelial duct opening ventrally, yellow secretion—were already described by Marcus (1953a). He noted similarities to the 'Blochmann's' gland in *Aplysia* but followed Guiart (1901) in simply naming it a 'pallial' gland. Salvini-Plawen (1973) highlighted the similarities to the Runcinacean 'pallial' or 'suprabranchial' gland; we confirmed this observation. In histological aspects, the gland of *Pluscula* resembles most the 'yellow' gland of

Fig. 5 a–f Semithin histological cross-sections of posterior body half. Dorsal side at top. **a** Overview at level of nidamental glands. **b** Detail of nidamental glands with interjected sperm package. **c** Ovarial follicles. **e** Most distal gonoduct and osphradium. **f** Yellow gland. **g** Caudal dorsal depression with shell 'remnant', insert: complete cross-section. *am* Ampulla, *an* anus, *au* auricle, *bc* bursa copulatrix, *bs* bursa stalk, *dg* digestive gland, *dgl* digestive gland lumen, *eg* egg, *fg1* albumen gland, *fg2* membrane gland, *fg3* short limb of mucus gland, *fg4* large limb of mucus gland, *fgl* female gland lumen, *ft* foot, *gd* gonoduct, *it* intestine, *oc* oocyte, *of* ovarial follicle, *osp* osphradium, *pc* pericardium, *shd* shell 'dimple', *shr* shell 'remnant', *sg* seminal groove, *sp* interjected sperm package, *ve* ventricle, *ygd* duct of yellow gland. *Bars* **a**, **c** 100 μm; **b**, **d**, **g** 50 μm; **e**, **f** 25 μm
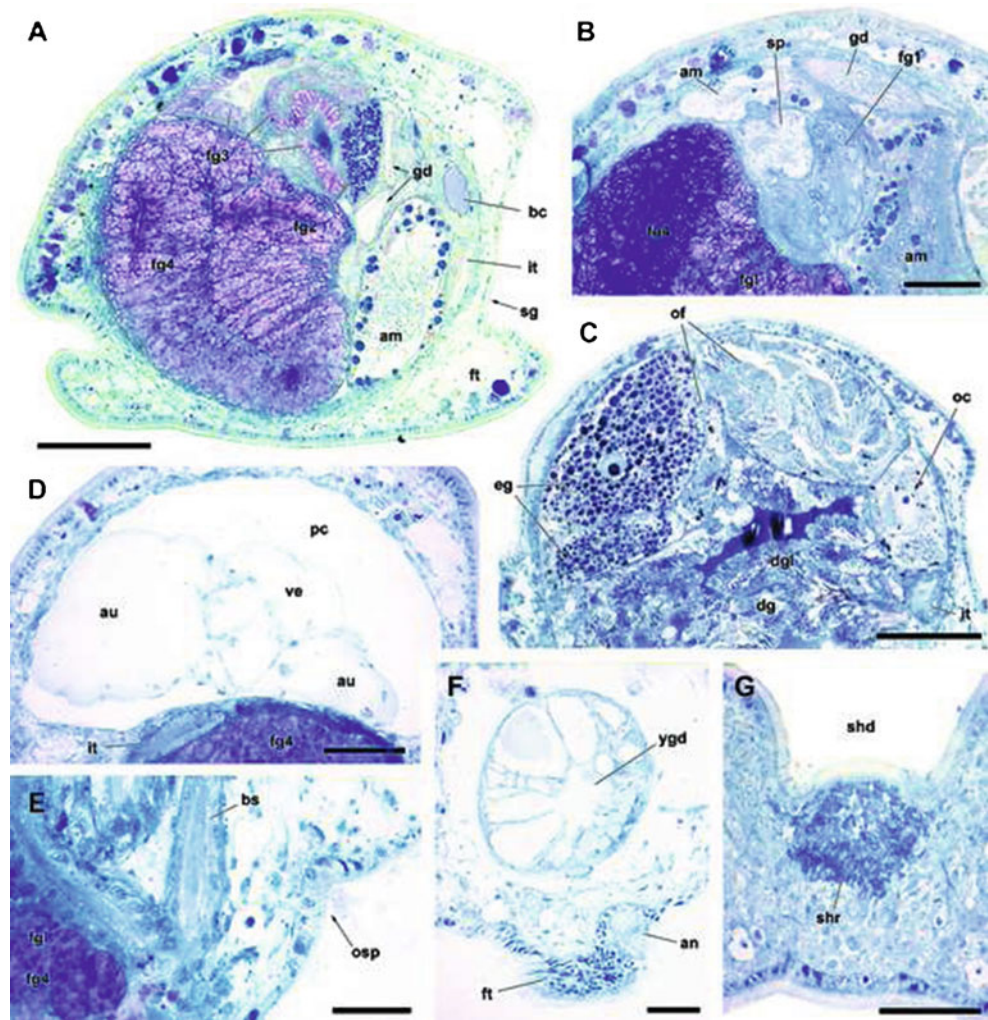
aglajids which Rudman (1972a, 1978) considered unique for that family. Dayrat and Tillier (2002) rejected the homology of yellow and purple/Blochmann's glands, and Blochmann's glands were coded as absent for aglajids by Wägele et al. (2006). Most other philinoids also show glands in the mantle cavity, but these are often groups of single subepithelial cells that do not open though a common epithelial duct and are therefore difficult to homologize. For example, the 'pallial' glands of some *Philine* species consist of a patch of cells that open separately into the mantle cavity (e.g., Challis 1969a; Rudman 1972b: 'posterior' gland; Guiart 1901: 'fossette glandulaire'). Nevertheless, a conspicuous yellow secretion was reported for *P. trapezia* Hedley, 1902 (Rudman 1998) and *P. caledonica* Risbec, 1951 (Risbec 1951; which might be the same species according to Rudman, 1998). Members of the Gastropteridae sometimes show a patch of dark-staining glandular cells surrounding the anus (Brodie et al. 2001; Klussmann-Kolb and Klussmann 2003); their additional large 'posterior pedal gland' is different in structure or in position (e.g., Gosliner 1994). Lemche (1956) reported the unicellular or multicellular 'Blochmann's' glands of *Cylichna*, positioned

dorsally in the mantle cavity roof and with an epithelial duct, to contain a secretion that is yellow in life but does not stain with methylene blue. *Scaphander lignarius* L., 1758, a species that produces a thick yellow fluid when disturbed (Guiart 1901), possesses single-celled Blochmann's glands that open through an epithelial duct (Perrier and Fischer 1911). Therefore, it seems that glands situated dorsally in the mantle cavity (or what is left of it) are present plesiomorphically in most philinoids, and persist in many or most other cephalaspideans and Euopisthobranchia. The aforementioned histological staining properties and position have also been reported for the Blochmann's gland of *Haminoea* by Wägele and Klussmann-Kolb (2005: Fig. 5d). Therefore, we regard the yellow gland of philinoglossids and aglajids to be a derived multicelled Blochmann's gland. The specific configuration may represent a synapomorphy of these two families. However, since the most recent molecular phylogenies never found a sistergroup relationship between the two families, the yellow gland might have been lost or modified in other philinoidean lineages, or may be a product of convergent evolution in philinoglossids and aglajids. Regarding the function of the aglajid gland,

Rudman (2001) assumed either an excretory or defensive function and observed the secreted substance to be toxic for annelids. Sleeper et al. (1980) identified the gland's secretions in *Navanax* as 'alarm pheromones', Cruz-Rivera (2011) observed an 'amber-coloured' secretion to repel potential fish predators.

Pluscula cuica thus matches other philinoglossans in the reduction of a distinct shell, although associated tissues are still present. The mantle cavity is also lost, but most organs and body openings found within the ancestral cephalaspidean mantle cavity are still present underneath the caudal overhang. Only the gill and current-inducing ciliated strips—typical for philinoidean mantle cavities (e.g., Rudman 1972b)—are lost completely, as is the case in all other meiofaunal slug lineages (Swedmark 1968; Arnaud et al. 1986). This loss of course indicates that respiration has to take place entirely through the body wall, as is supported by Bartolomaeus' (1997) observation of numerous subepidermal blood sinuses in *Philinoglossa helgolandica* Hertling, 1932.

Circulatory and excretory systems

Our findings on the circulatory and excretory systems of *Pluscula cuica* correspond well to the original description (Marcus 1953a). The heart is located slightly right of the midline, and consists of a wide auricle posterior and slightly left of the ventricle, indicating that *Pluscula* is almost completely detorted. This organization is in general agreement with Bartolomaeus' (1997) ultrastructural study on the heart and kidney of *P. helgolandica* which showed that the valve is described to consist of only a single, flattened cell. Judging from our histological sections, there appear to be more nuclei in the valve of *Pluscula*. As described by Marcus (1953a), the kidney is largely horseshoe- or 'u'-shaped and consists of a slim part running from the pericardium to the left, and a more voluminous part curving back to the nephropore at the right body side. The parts of the kidney appear very similar in histology; we were not able to detect ciliation in the proximal part described for *P. helgolandica* by Bartolomaeus (1997).

Digestive system

The digestive system of *Pluscula* conforms well to the original description and the general philinoglossan organization. Described differences among the genera can be found in the presence of denticles on the first lateral teeth, possibly the presence of a vestigial crop in *Pluscula* and in the form and dimensions of the digestive gland.

Nearly all philinoglossids are described with a long and curved pharynx similar to that of *Pluscula*, with the radula situated far posterior (Hertling 1932; Marcus and Marcus 1954; Marcus 1959). Our material suggests that the anterior part of the pharynx and especially the posterior oral tube are rather expandable due to the presence of longitudinal folds.

Chitinous jaw plates present in Euopisthobranchia are secondarily lost in many Philinoideans (Burn and Thompson 1998), including philinoglossids. Jaws are present only in some philinids and allgastropterids (Rudman 1972b; Gosliner 1980, 1989), therefore jaws were lost multiple times convergently. All philinoglossans possess a radula (formula given as $n \times 3.0.3$ or $2.1.0.1.2$) that especially resemble philinids and gastropterids in tooth form (see Gosliner 1994). Since reduction of the rhachidian tooth row has occurred separately in all other philinoid families, it is therefore hardly useful for phylogenetic comparison with philinoglossans (see Gosliner 1980; Rudman 1972b). The first lateral teeth of *Pluscula*, *Abavopsis*, *Philinoglossa praelongata* and *P. marcusi* are described without smaller denticles along the masticatory border; however, denticles of this size might be hard to detect without SEM studies and their number also depends on the size of specimens (see Salvini-Plawen 1973; Challis 1969b). Therefore, 'absence of denticles' in the literature might not always be a useful taxonomic character in philinoglossans, as is exemplified by *Pluscula*: neither Marcus (1953a) nor our light microscopical examination of sectioned material and separated radulae revealed denticles, but Marcus and Marcus (1954) mention about 20 denticles per tooth in later collected material. Comparative re-examination using scanning electron microscopy might be needed to reveal if denticulate teeth occur consistently in any philinoglossan, or if intraspecific plasticity reduces the taxonomic value of this character, as is known for some other marine gastropods (e.g., Padilla 1998; Reid and Mak 1999). Following the pharynx, *Pluscula cuica* shows a slightly dilated esophagus where most other philinoideans have an unarmed crop (Aglajidae, e.g., Rudman 1974, Gastropteridae: Gosliner 1989) or a gizzard armed with cuticular plates to grind up hard-shelled food (many Philinidae: Rudman 1972b). Neither crop or gizzard are described for other philinoglossans, but the structure found in *Pluscula* may represent vestiges of the ancestral condition, if not an artifact. A gizzard armed by cuticle was regarded as a synapomorphy of Euopisthobranchia (Jörger et al. 2010a), but spines or calcareous plates are reduced secondarily in many philinoideans (Burn and Thompson 1998).

Pluscula (and *Sapha*) do not possess a histologically distinct stomach between esophagus and digestive gland, in contrast to *Abavopsis* and *Philinoglossa*, which are described with a small and smooth-walled stomach (Salvini-Plawen 1973). In *Pluscula*, the pale yellow digestive gland is a single sac and located anterior to the gonad in mature specimens. The sloping rear face of the digestive gland—visible in living specimens—might be a useful diagnostic character for *Pluscula*, and was also observed in an undescribed species from Belize (K.M. Jörger, Munich, personal observation). In all other species the digestive gland extends almost to the end of the body cavity. *Sapha* and *Abavopsis* possess a single digestive gland (Marcus 1959; Salvini-

Plawen 1973); in *Philinoglossa* there are two tubular branches, one of which is long, coiling, and ventral to the gonad (Hertling 1932; Marcus and Marcus 1954; Salvini-Plawen 1973). The latter case resembles other philinoids that possess more than one digestive gland, e.g., *Philine exigua* (Challis 1969a; Martínez et al. 1993). In all philinoglossans, the intestine emerges from the stomach/ digestive gland anterodorsally and curves along the right body side; the anus is posteriomedian. Only in *Abavopsis* the intestine is described to emerge more on the left, running underneath (!) the digestive gland for much of its course (Salvini-Plawen 1973). The funnel-like extension of the proximal intestine into the digestive gland lumen was found only in the reconstructed specimen and may be an artifact, since it is not reported for other philinoglossans species.

There are no reports of philinoglossan food sources, although Marcus and Marcus (1954) mention 'a large diatom' in the intestine of *P. remanei* Marcus and Marcus, 1958. The lack of distinct cuticular armament in the gut implies that food is not hard-shelled. Radular morphology, coupled with the thin pharyngeal cuticle and infolding of the (?dilatable) preradular digestive tract, may hint at a carnivorous habit of philinoglossans on soft-bodied prey. Although predation was not observed directly, co-occuring acochlidians extracted from sand samples disappeared from Petri dishes when kept with philinoglossans over night and thus may be a possible food source, at least under lab conditions (own observations). Carnivory would be consistent with the general condition in Philinoidea.

Central nervous system

One reason to argue for a basal phylogenetic position of *Pluscula cuica* within Philinoglossidae, or for separation from the latter in its own family, was the supposed "primitiveness" of the cerebral nerve ring and the visceral nerve cord. This was based on the supposed separation of cerebral and pleural ganglia (Marcus 1953a, 1959; Salvini-Plawen 1973) and also the presence of five distinguishable ganglia on the visceral nerve cord (albeit four of them closely allied, forming two pairs; Marcus 1953a). Reexamination of the nervous system, however, shows that neither is the case. Free pleural ganglia in *Pluscula* were identified originally by Marcus (1953a) lateral to the cerebral ganglia, with connectives to the latter and the pedal ganglia. This is a misobservation, since cerebral and pleural ganglia form fused cerebropleural ganglia as is evident from semithin histological sections and visible on the 3D model. As other philinoglossans, *Pluscula* has cerebropleural ganglia showing characteristic double connectives to the pedal ganglia. Marcus' laterally situated 'pleural' ganglion therefore is most likely the (posterior) rhinophoral accessory ganglion; however, the reported connective of these laterally situated ganglia to the pedal ganglion does not exist. This unusual

lateral-pedal connective was also described for the 'lateral' ganglia of *Philinoglossa remanei* and *P. praelongata* (Marcus and Marcus 1954; Salvini-Plawen 1973). It should be critically reinvestigated whether this connective presents a genuine structure.

The presence of five ganglia on the visceral cord has been proposed as a synapomorphy of Euthyneura (=Pentaganglionata, Haszprunar 1985, 1988), although most taxa possess a lower number of separate ganglia that have been interpreted as the result of various stages of ontogenetic fusion. Dayrat and Tillier (2000) challenged such a scenario claiming that there are very few reliable examples of euthyneurans showing a pentaganglionate condition, i.e., just six genera, of which two belong to basal heterobranchs according to molecular data (see Schrödl et al. 2011a). *Pluscula* was overlooked as a pentaganglionate candidate; if confirmed, it would be the only cephalaspid reliably showing five ganglia on the visceral loop. Our results, however, demonstrate that mature *Pluscula* possess only three ganglia on the visceral nerve cord. These three ganglia correspond well to the single ganglion and two closely aligned pairs mentioned by Marcus (1953a), although our material shows more than superficial fusion. The visceral nerve cord of *Pluscula* is not fundamentally different from that of other philinoglossans, since all other species are described with three ganglia, except for *P. praelongata* which Huber (1993) reinvestigated and reported four (although his Fig. 10 shows only three).

Cerebral nerves and sensory organs

Pluscula cuica possesses a set of four paired cerebral nerves (plus a single nerve on the left side) that correspond well to the nerves found in previous investigations of other cephalaspidean species (Faller et al. 2008; Staubach et al. 2008). Following the nomenclature of nerves identified by the previous authors and Huber (1993) in other heterobranchs, we identified an oral nerve (anteromedian), the labiotentacular nerve (basally branched, with one large and several small extra ganglia), the rhinophoral nerve (possibly with a double root, basally branched with two large extra ganglia), and a small nervus clypei-capitis (head-shield nerve). The single median nerve extending from the left cerebral ganglion could not be identified, and a corresponding nerve on the right side was not detected either. The finding of a vestigial head-shield nerve (n. clypei-capitis) in *Pluscula* is important since it suggests an ancestral presence and secondary reduction of a functional cephalaspidean headshield in philinoglossans. Most cephalaspideans possess an elaborate nervus clypei-capitis that innervates the posterior part of the head-shield (e.g., Staubach et al. 2008); this nerve is less branched in other heterobranchs, if identified at all (Huber 1993). Reduction of an externally discernible head-shield is thus confirmed as one of the synapomorphies of philinoglossans (Arnaud et al.

1986). Only *Abavopsis latosoleata* shows a slight transversal groove indicating remainders of a separate head-shield (Salvini-Plawen 1973), and previously only this genus was shown to possess a thin nervus clypei-capitis branching from the base of the rhinophoral nerve (Huber 1993). If confirmed, a loss of the headshield nerve in *Philinoglossa* (shown by Huber 1993) and *Sapha* might represent a synapomorphy uniting these genera.

Pluscula cuica is unusual among philinoglossans in that it lacks eyes, which appears to be an apomorphy of the species. In *Abavopsis* and *P. praelongata,* the eyes are innervated through a branch of the large labiotentacular accessory ganglia (Salvini-Plawen 1973). Among meiofaunal slugs, loss of eyes is found convergently among several taxa (Swedmark 1971), e.g., among rhodopemorphs (own observation), pseudovermids (see Urgorri et al. 1991) and some acochlidians (Marcus 1953a).

We are not aware of literature mentioning the paired 'blisters' embedded in the pedal ganglia next to the statocysts. They are not present in *Philinoglossa praelongata* (own observation). The structures might represent single specialized cells. If not for their position next to the statocysts, one might confuse the structures with the vestigial, unpigmented eyes found, e.g., in some acochlidians (see Challis 1968; Neusser et al. 2011a).

Accessory ganglia

Accessory ganglia anterior and lateral to the cerebropleural ganglia are described for all philinoglossans examined in detail, but nomenclature and proposed innervation patterns differ considerably in the descriptions (e.g., Marcus 1953a, 1959; Salvini-Plawen 1973; Huber 1993). In all cases there appear to be large ganglia (lateral and anterolateral to the cerebropleural ganglia) and distinctly smaller ones (mostly anterior and more median). In *Pluscula*, one large rhinophoral ganglion was identified originally as the pleural ganglion (see above); five further 'precerebral' ganglia were described on both branches of the labial nerve (Marcus 1953a). In *Sapha*, there are paired large 'Hancock's' and 'olfactory' ganglia, and pairs of small 'labial' and 'prepedal' ganglia (Marcus 1959); innervation of these ganglia was not described. *Abavopsis* was originally described without accessory ganglia (Salvini-Plawen 1973), but Huber (1993) showed that there are two large ganglia on each rhinophoral nerve and one large and one small on each labiotentacular nerve, similar to the condition found in *Pluscula*. *Philinoglossa praelongata* was described originally with small anterior 'accessory' ganglia and two large ganglia innervating the Hancock's organs: one 'olfactory' ganglion (with the two connectives to the cerebropleural and pedal ganglia as originally and falsely described for *Pluscula*; = accessory rhinophoral ganglion?) and one 'labial' ganglion (also innervating the eye; = large labiotentacular

ganglion?) (Salvini-Plawen 1973). Except for the double connective, this configuration largely agrees with Huber's (1993) examination of the same species. A connective between the pedal and a large 'precerebral' ganglion was again described for *Philinoglossa remanei* by Marcus and Marcus (1954); this ganglion also innervates the Hancock's organ together with two 'olfactory' ganglia, besides smaller 'labial' ganglia. The number of large ganglia in *P. remanei* (two or three) is not entirely clear. Summarizing the literature and homologizing with the ganglia found in *Pluscula*, the following general pattern of innervation of the accessory ganglia appears to be present in all philinoglossans: there is one accessory rhinophoral ganglion in *Sapha* and *Philinoglossa praelongata*, and two in *Pluscula* and *Abavopsis*. These and the large accessory labiotentacular ganglion innervate the posterior and anterior parts of the Hancock's organ, as is postulated or observed for numerous cephalaspideans (e.g., Huber 1993; Mikkelsen 1996; Staubach et al. 2008). A variable number of smaller accessory labiotentacular ganglia innervate the lip and/or oral tube.

Additional, accessory ganglia innervated by cerebral nerves are characteristic features of meiofaunal slugs. These structures are described for rhodopemorphs (Salvini-Plawen 1991), pseudovermid nudibranchs (Ev. Marcus 1953a; Huber 1993), the sacoglossan *Platyhedyle* (Rückert et al. 2008), microhedylacean acochlidians (e.g., Neusser et al. 2006) and the limnic hedylopsacean *Tantulum* (Neusser and Schrödl 2007). Among Cephalaspidea, only philinoglossans and *Philine exigua* (Challis 1969a) show accessory ganglia. Wherever examined, these accessory ganglia are innervated by the rhinophoral and labiotentacular nerves (as in *Pluscula*). Accessory ganglia are often histologically distinct in lacking a separation into cortex and medulla (Neusser et al. 2006). Marcus (1953a) specifically states that this is not the case in *Pluscula cuica* (in contrast to the acochlidian *Ganitus evelinae* described in the same paper). Our material shows that the neurons in the accessory ganglia of *Pluscula* are considerably smaller than those in the other ganglia, making identification on histological sections possible at a glance. This is in contrast to the accessory ganglia of acochlidians that differ in overall organization but not in neuron size (as mentioned above). The function of the conspicuous accessory ganglia of meiofaunal heterobranchs has so far been a matter of speculation. Haszprunar and Huber (1990) argued that additional neurons were needed in small-sized ganglia to help mediating 'essential activities'. However, they also noted that miniaturized slugs that are not meiofaunal, e.g., runcinids or the nudibranch *Vayssierea*, do not show these accessory ganglia (e.g., Huber 1993; Baba 1937) and that the evolution of accessory ganglia is therefore linked to the mesopsammic habitat. Since the accessory ganglia are invariably found associated with sensory nerves, they might rather reflect the need of additional nervous capacity in this three-dimensional interstitial living space, as was argued by Jörger et al. (2008). The development

of large accessory ganglia innervating the Hancock's organs may imply comparatively enhanced chemosensory or tactile capabilities, involved in trailing chemical cues or for simply finding the easiest way to push through the complex three dimensional pore-spaces of the interstitial habitat.

Osphradium

Pluscula cuica is the so far only meiofaunal slug demonstrated to possess an osphradium with an associated ganglion. Originally, a posterior 'genital' ganglion close to the female genital opening was described for *Pluscula* and *Sapha* (Marcus 1953a, 1959), but innervation patterns were not observed. In *P. remanei*, Marcus and Marcus (1954) assumed innervation by the visceral nerve. In *Abavopsis*, a possibly similar ganglion is located at the posterior end of the copulatory organ (Salvini-Plawen 1973). Our material of *Pluscula* confirms the presence of the ganglion next to the genital opening and also shows innervation of a small pit resembling a small osphradium in histology (ciliated pit with higher, unstained, columnar cells; see Edlinger 1980) and position (right body side, close to organs and body openings plesiomorphically situated in a mantle cavity). We therefore regard this posterior ganglion to be homologous to the osphradial ganglion of other heterobranchs. In this case the ganglion should be innervated by the nerve extending from the combined right parietal and supraintestinal ganglion (e.g., Haszprunar 1988) and not the visceral nerve which leads into the same general direction. A chemosensory osphradium has not been reported for any other meiofaunal slug. Many acochlidians possess an osphradial ganglion, but an osphradium was detected only in the secondarily large-bodied *Strubellia* and *Acochlidium* (Brenzinger et al. 2011a). Osphradia are likely present in many meiofaunal slugs with an associated ganglion, but in these cases the sensory epithelium has been reduced to only few sensory cells. Presence of sensory areas in other species bearing osphradial ganglia needs reinvestigation.

Reproductive system

The reproductive system of *Pluscula cuica* unites usual and thus plesiomorphic philinoid cephalaspidean features with those that appear highly derived but typical for meiofaunal slugs. The hermaphroditic gonad of adult *Pluscula* is not divided into distinct female and male follicles save for the medial and strictly male follicle. The latter was also described by Marcus (1953a), but interpreted as an autosperm ampulla rather than part of the gonad. Contrary to *Pluscula*, *Sapha* and *Philinoglossa remanei* have strictly female acini located either at the left side or ventral of the strictly male ones, respectively (Marcus and Marcus 1954; Marcus 1959). Data on *Abavopsis* are not conclusive. Spatial separation of gamete production is a feature commonly found in meiofaunal slugs (Swedmark

1968): *Rhodope* shows a consecutive separation of male and female ovotestis follicles (Brenzinger et al. 2011b), some meiofaunal acochlidians have separate ovaries and testes (Morse 1976) or are completely gonochoric (Challis 1968; Schrödl and Neusser 2010). The meiofaunal *Philine exigua* has some follicles that produce either only one type of gamete besides follicles that produce both (Challis 1969a).

Philinoideans generally possess three different sperm storing structures (besides one associated with the copulatory organ): a proximal ampulla for autosperm, a receptaculum seminis for long term storage of allosperm, and a distal bursa copulatrix for allosperm storage and/or lysis (e.g., Gosliner 1994; Mikkelsen 1996). Identification of these structures according to their relative positions rather than histology or a combined approach is advocated (Gosliner 1994; Valdés et al. 2010), but may be a preconception that misses actual structure, homology and function (e.g., Mikkelsen 1996; Wägele and Willan 2000). Our histological data suggests that *Pluscula* possesses a stalked, sac-like ampulla that is unusual in several aspects: first, it is extremely large and splits off an unusually long part of gonoduct that is located between gonad and nidamental glands (instead of being a widening close to the gonad). The ampulla reaches far anterior, but it opens to the gonoduct at its posterior end. Second, the ampulla shows an unusual but distinct histology with large (?lipid) droplets covering the wall, instead of being a thin-walled sac conforming to the gonoduct in histology (see Gosliner 1994). A proximal ampulla is described for all philinoglossan genera; it is also sac-like but smaller in *P. remanei* (Marcus 1953a; Marcus and Marcus 1954), but tubular in *Sapha* (Marcus 1959).

Pluscula does not show a distinct receptaculum seminis: this organ usually follows the ampulla closely and would be identifiable by spermatozoa embedded into the muscularly lined wall with their heads (e.g., Beeman 1977). No such structure is found in the material examined herein, and no receptaculum is described for any other philinoglossans. Loss of a distinct proximal receptaculum seminis may represent a synapomorphy of philinoglossans, since it is present in other philinoidean groups (e.g., Rudman 1972a, b; Gosliner 1980, 1989).

We interpret the distal stalked sac, filled with pink secretion and branching from the gonoduct close to the genital opening, to be a bursa copulatrix. Marcus originally described this structure in *Pluscula* as a 'spermatheca or receptaculum seminis that contains spermatozoa' (1953: p 180); he also describes a 'red and blue' staining secretion. This histological character is typical for the allosperm-digesting bursae, but not for a receptaculum according to newer terminology (Beeman 1977; Valdés et al. 2010). No other philinoglossan is described with a similar structure, but a bursa with at least temporary gametolytic function is present in most other philinoids and may represent a plesiomorphic character in *Pluscula*.

The pocket containing spermatozoa between the membrane and mucous glands in one examined specimen is most likely not a permanent feature. It may be a received package of allosperm or a spermatophore, a temporary fertilization chamber, or a package of autosperm on its way out.

The three parts of the female gland mass of *Pluscula* correspond well to the albumen, membrane and large mucous glands of most other 'opisthobranchs' (Gosliner 1994; Klussmann-Kolb 2001), but comparison to other philinoglossans is not straightforward due to ambiguous literature. *Philinoglossa remanei* has a 'protein' gland and sac-like mucous glands (Marcus and Marcus 1954); the nidamental glands of other species are not described in further detail. In *Abavopsis*, the nidamental glands are situated posteriorly as in *Pluscula*, but are apparently followed by a long distal gonoduct part leading to the anteriorly shifted genital opening (Salvini-Plawen 1973). In *Philinoglossa* and *Sapha*, the distal gonoduct is short since the female glands are also shifted towards the genital opening (Marcus and Marcus 1954; Marcus 1959). This situation differs from that of *Pluscula* and other philinoideans and may be a synapomorphy of a *Philinoglossa*/*Sapha* clade.

The female genital opening in *Pluscula* is close to the posterior end of the body—as in other philinoids—showing its affiliation with the ancestral mantle cavity (Burn and Thompson 1998). In the remaining philinoglossans the opening is in the anterior right third, e.g., at the posterior border of the head-shield in *Abavopsis* (Salvini-Plawen 1973); therefore, the seminal groove that is present in philinoglossans is generally short compared to that of *Pluscula*. At least in *Pluscula*, there is a gap between the seminal groove and the male genital opening. Marcus (1953a) identified acidophilous glands along the rim of the anterior sperm groove in mature individuals, and assumed a role in guiding spermatozoa. We were able to identify additional glands in the foot at this position, although they seem to open through the foot sole and not the sperm groove.

Pluscula cuica possesses a sac-like cephalic copulatory organ that contains several histologically separable parts. Marcus (1953a) originally identified the following elements (from anterior to posterior): an epidermal pouch, a narrow and tubular penis, followed by a short tubular prostate, and a bulbous 'seminal vesicle'. Our material shows that the penis consists of a rather short ring-like structure at the base of the epidermal pouch which is followed by a tube with strong subepidermal circular muscles. The posterior part is histologically uniform becausethe prostate and its autosperm-storing end are confluent, instead of forming a distinct 'seminal vesicle'. In *Sapha*, the copulatory organ was also described to consist of four parts (Marcus 1959), but with a different order: following a distinct penial papilla, there is a long prostate and then a sphincter-like muscle (and not vice versa), the muscle closing the large spherical seminal vesicle. Marcus

and Marcus (1958) show a similar configuration in *P. helgolandica*, but mention the short part anterior to the 'seminal vesicle' to be of glandular nature, not muscular. In *P. remanei* and *Abavopsis*, the copulatory organ is described as a simple, bag-like structure with variable orientation, even looping around the oral tube (Marcus and Marcus 1954; Salvini-Plawen 1973). It remains unclear whether the copulatory organ is truly less elaborate in the latter taxa compared to the condition found in *Pluscula*. Nevertheless, the sac-like copulatory organ of philinoglossans in general appears to differ from that of other philinoideans in being less elaborate, probably due to size constraints. Judging from histological examinations, there is no true eversible papilla (perhaps excepting *Sapha*) but only a slightly prominent ring, and there never is the cuticular armament found at least in some groups, e.g., Gastropteridae (Anthes and Michiels 2007a, b). Functionally more important, in philinoglossans there is no separate posterior-leading vas deferens ("ejaculatory duct" according to Mikkelsen 1996) leading directly to the prostate as e.g., in *Philine* species (Rudman 1972b); therefore, autosperm have to enter and exit the copulatory organ via the same opening. This two-way configuration is more similar to what is found, e.g., in the spermatophore-producing *Runcina* species (Kress 1985).

Sperm transfer by a "kiss"?

Rather than anterodextrally as in most cephalaspideans, the male genital opening of *Pluscula* is situated frontally at the head. It is joined to the anterior oral tube, as was also observed by Marcus (1953a). The same condition is reported for *P. helgolandica* (Marcus and Marcus 1958), *Sapha* (Marcus 1959) and *Abavopsis* (Salvini-Plawen 1973). This means that the copulatory organ of philinoglossans has to be everted through the mouth during copulation. It seems that philinoglossans have taken to the extreme a trend that is found in Aglajidae and Gastropteridae (see Anthes and Michiels 2007a, b), where the male genital opening is shifted to underneath the anterior side of the headshield. This is in contrast to other philinoideans that have it located more on the right side of the head (e.g., Rudman 1972b), as is the plesiomorphic condition for cephalaspideans. More specifically, the male genital opening inside the mouth is also found in the meiofaunal acochlidian *Pontohedyle milaschewitchii* Kowalevsky, 1901; this aphallic species glues spermatophores indiscriminately onto a partner's epidermis (Jörger et al. 2008, 2009). In the meiofaunal *Philine exigua*, the opening appears also to be more anterior than in other, burrowing or benthic members of the genus (Challis 1969a). The extreme anterior shift may therefore be another adaptation particular of meiofaunal groups, facilitating sperm transfer within the limited space and dynamics of sand interstices (Swedmark 1964): in an animal

moving between sand grains, it is the anterior face that touches a partner most readily. Sperm transfer would be possible by a simple "kiss" on a quickly passing partner's epidermis (in the case of hypodermal injection or dermal insemination), or on the genital opening in species that copulate. This latter head-to-tail mode of copulation can be suggested for *Pluscula* because of the opposite positions of the male and female genital openings, and because sperm transfer by a trailing 'male' is known to take place in a number of other philinoideans (e.g., Rudman 1972a). However, since the other philinoglossan genera have also shifted the female genital opening anteriorly, copulation in these genera could more be bilateral or sequential, but also more head-to-head and thus again less space-consuming.

Autosperm transport through the hemocoel?

Marcus' (1953a) original description of *Pluscula cuica* suggests a highly peculiar mode of autosperm transport, probably unique among gastropods: on their way between the gonad's follicles and the sperm-storing part of the copulatory organ, sperm were hypothesized to move directly through the hemocoel, and not along the gonoduct and external ciliated groove. This was concluded because (1) apparently all 'mature' specimens examined by Marcus showed numerous spermatozoa free in the body cavity, with the highest density between gonad and copulatory organ, and (2), the external ciliated groove was found to disappear before connecting to the copulatory organ, implying that its original function as a conveyor of autosperm was lost.

We can confirm the peculiar lack of a continuous sperm groove in *Pluscula*, although the gap could be explained by the presence of sensory epithelium (Hancock's organs) in this place (Fig. 1a). Since the lateral furrow itself is quite narrow, it might still have sufficient capability in guiding sperm towards the mouth. Furthermore, there are additional glands below the end of the sperm groove which Marcus (1953a) hypothesized to facilitate a further passage of sperm by producing 'protective secretions' (1953: p 181). The lack of a continuous sperm groove might be a consequence of an overall beneficial apomorphic anterior shift of the copulatory organ. A gap in ciliation may not be much of a hindrance to sperm transport: spermatozoa are known to be capable of moving along the epidermis of species without such a groove [Karlsson and Haase 2002 in the nudibranch gastropod *Aeolidiella*; Brown (1979) on *Colpodaspis thompsoni*]. Since our specimens examined were mature hermaphrodites and none of them contained free spermatozoa in the hemocoel (as would be expected assuming internal autosperm transport) we conclude that sperm is conveyed externally via the sperm groove, as usual.

How then to explain Marcus' observation of hemocoelic spermatozoa in *Pluscula*? If autosperm, it could be squeezing

or fixation artifact, or it could have been allosperm. In other meiofaunal slugs, a proportionally common mode of sperm transfer is by hypodermal injection or dermal insemination: it was suggested for species of *Rhodope* (Brenzinger et al. 2011b) and was observed in the microhedylacean acochlidians *Pontohedyle* and *Ganitus evelinae* Marcus, 1953a (Jörger et al. 2009; Marcus and Marcus 1954). In these generally aphallic species, sperm are transferred through the epidermis; at least in *Pontohedyle* this happens by lysis of epidermal cells induced by the dermally applied spermatophore (Jörger et al. 2009). After dermal insemination, the spermatozoa move through the body cavity and fertilization supposedly takes place somewhere inside the gonoduct or directly in the gonad. Explaining Marcus' (1953a) observation of hemocoelic sperm in *Pluscula cuica* in a similar way is, however, inconsistent with the presence of a distal bursa copulatrix in the species. Such an allosperm storage organ is usually present only in copulating species, or in non-copulating species that may inject spermatozoa directly into the (large) bursa using a copulatory stylet (e.g., the acochlidian *Pseudunela*; Neusser et al. 2009b). Since hemocoelic spermatozoa have never been reported in other philinoglossans, their occurrence should be critically reinvestigated in other species.

Origin of the Philinoglossidae

The advent of molecular systematics cast doubt on long-held beliefs in euthyneuran topologies, and studies using multi-locus markers started to change our concepts of their evolution (e.g., Göbbeler and Klussmann-Kolb 2011; Jörger et al. 2010a). The backbone topology of a "new euthyneuran tree", with Nudipleura basal to the common clade of Euopisthobranchia and panpulmonates—as summarized by Schrödl et al. (2011a)—was supported by recent phylogenomic data (Kocot et al. 2011; Smith et al. 2011), and is also compatible with a recent molluscan phylogenetic study based on housekeeping genes (Vinther et al. 2011). In contrast, the traditional concept of monophyletic Opisthobranchia and Pulmonata is contradicted by all phylogenomic and other approaches that include nuclear rather than mitochondrial genes.

Rather than being basal opisthobranchs, the Cephalaspidea in a modern sense (sensu Malaquias et al. 2009) form one of several clades of the so called Euopisthobranchia (Jörger et al. 2010a) among tectipleuran Euthyneura (Schrödl et al. 2011a). Philinoglossans lack the major euopisthobranch synapomorphy, a cuticularized gizzard. Having a large body-shield rather than a head-shield, a posterior mantle cavity, and a simple, frontal copulatory organ they somewhat resemble similarly small-sized runcinids. However, molecular data clearly indicate that philinoglossans are cephalaspideans in the strict sense (Jörger et al. 2010a; Göbbeler and Klussmann-Kolb 2011). The prepharyngeal nerve ring combined with monaulic genital system qualifies Philinoglossidae as Cephalaspidea

sensu Malaquias et al. (2009), and the presence of a secondarily modified head-shield innervated by the nervus clypei-capitis fits with the placement into a higher, non-diaphanoidean clade. Having a narrow radula, a carnivorous gut type without cuticle and gizzard plates and a slender, at least externally shell-less body points towards a placement among philinoidean lineages. In fact, both multi-locus analyses with broader taxon sampling (Malaquias et al. 2009; Göbbeler and Klussmann-Kolb 2011) identify a philinoidean clade of *Philinoglossa* and Gastropteridae as sister to Aglajidae plus Philinidae, with Scaphandridae as outgroup. At the current state of knowledge, possible shared characters of a gastropterid/philinoglossid clade may be a comparatively short headshield and the anterior shift of the copulatory organ. A philinoglossid/aglajid clade on the other hand would be supported by the presence of a spherical yellow gland and the loss of jaws. Molecular hypotheses on the origin of Philinoglossidae within Philinoidea thus are consistent with morphological evidence discussed herein and by Salvini-Plawen (1973), although the exact position remains unclear. Nevertheless, a previously proposed higher category, i.e., ordinal Philinoglossacea Thiele, 1931, is no longer required.

We show that previously discussed "primitive", potentially progenetic or at least aberrant features such as separate pleural and cerebral ganglia, a pentaganglionate visceral loop, and hemocoelic autosperm transfer in *Pluscula* were due to misobservations or artifacts. A gizzard with three plates that is characteristic of ancestral, non-carnivorous cephalaspideans including philinoidean Scaphandridae and Philinidae is absent in most Aglajidae, Gastropteridae (Rudman 1978; Gosliner 1989), and likely carnivorous philinoglossans. This supports their independent origin from mesopsammic *Philine exigua* as indicated by molecular analysis (Jörger et al. 2010a). We propose that philinoglossans are small-sized, though not obviously pedomorphic invaders of mesopsammic spaces, evolving a detorted streamlined body, precerebral accessory ganglia, a frontal, potentially unilateral mode of sperm transfer, losing and modifying allosperm receptacles, reducing the ancestral shell, and reducing and modifying the mantle cavity and associated organs. All these traits are adaptive and synapomorphic for Philinoglossidae, but have evolved convergently in interstitial members of other heterobranch lineages. The conspicuous yellow gland found in *Pluscula* and other philinoglossans can be roughly homologized with similar glands in other philinoidean lineages (especially Aglajidae), but limited comparative histological knowledge inhibits definite conclusions.

Within Philinoglossidae, the case of *Pluscula cuica* showing a number of morphological plesiomorphies that support its basal position among philinoglossans is weakened. We could not find any shell, but putative vestiges of shell-forming tissue at most. An osphradium, the vestigial crop and the comparatively elaborate copulatory organ described herein might be

further plesiomorphies but need comparative reinvestigation in the other genera. Stronger evidence supporting a basal position are the retained posterior position of the female genital opening and the presence of a putative bursa copulatrix. None of these features was described from other philinoglossans. If confirmed, their apparent absence might be a synapomorphic loss, indicating that *Pluscula* is the most basal branch of Philinoglossidae, as had been assumed by previous authors (Marcus 1953a; Salvini-Plawen 1973). In conflict with this scenario are the putative retention of parapodia and an at least temporarily detectable separation of the head-shield from the rest of the notum in *Abavopsis*, and presence of two digestive gland branches in *Philinoglossa* species. Both parapodia and nervus clypei-capitis are more developed in *Abavopsis*, but remainders are still detectable in *Pluscula*. We suggest that a separate family Plusculidae for *Pluscula cuica* as established in the literature (e.g., Bouchet and Rocroi 2005) is no longer warranted.

Pluscula cuica can be distinguished externally from all other known philinoglossans by the lack of eyes, the dimple in the dorsal side of the caudal overhang, the presence of only a single digestive gland with a sloping posterior face. So far identified internal features include aforementioned plesiomorphies, and possibly the presence of the paired 'blister' cells next to the statocysts.

The remaining Philinoglossidae are united by further reductions (shell-associated tissue, bursa copulatrix) and shared characters (anterior shift of the female genital opening). *Philinoglossa* appears to be most derived (vermiform, tail-end glueing, simple copulatory organ, lateral separation of ovotestis follicles; Salvini-Plawen 1973) but shows two digestive gland lobes (cephalaspidean plesiomorphy). This highlights the continuing lack of comparable microanatomical data on the philinoglossans. Some current datasets, such as the denticulation of the lateral radula teeth as a criterion for species delimitation, should be reviewed (Salvini-Plawen 1973). The origin of monophyletic Philinoglossidae from a presumed gasteropterid—or aglajid-like ancestor—and the evolutionary scenario proposed herein with more or less successive adaptation to meiofaunal environment, should be further investigated. An integrative approach combining more comprehensive molecular datasets with additional morphological data seems most promising to evaluate proposed homologies and traits of evolution.

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3. RESULTS

Chapter 6

Ornelas-Gatdula E, Camacho-García Y, Schrödl M, **Padula V**, Hooker Y, Gosliner TM & Valdés Á (2012) Molecular systematics of the `*Navanax aenigmaticus*´ species complex (Mollusca, Cephalaspidea): coming full circle. *Zoologica Scripta* **41**: 374-385.

Molecular systematics of the 'Navanax aenigmaticus' species complex (Mollusca, Cephalaspidea): coming full circle

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Ornelas-Gatdula, E., Camacho-García, Y., Schrödl, M., Padula, V., Hooker, Y., Gosliner, T. M. & Valdés, Á. (2012). Molecular systematics of the 'Navanax aenigmaticus' species complex (Mollusca, Cephalaspidea): Coming full circle. —*Zoologica Scripta*, *00*, 000–000.
Molecular evidence from the mitochondrial COI and 16S genes and the nuclear H3 gene indicate that the traditionally recognized cephalaspidean sea slug species *Navanax aenigmaticus* consists of three deeply divergent lineages with disjunct ranges in the eastern Pacific, western Atlantic and eastern Atlantic. Each of these allopatric lineages is highly variable in colour and body size, which hampers identification of some possible consistent differences between them. Some conchological differences between the three lineages seem to be correlated with the groupings resulting from the analyses of molecular data, but the results of the morphological studies are inconclusive. Because of the presence of well-supported divergences and molecular synapomorphies, these lineages are herein considered to be three separate cryptic species. A review of the literature and available type material was conducted to determine the valid name for each of the three species. In other to promote nomenclatural stability, the oldest name with a description that allows a positive identification was selected over older, taxonomically ambiguous names. The conclusion of this revision is that the valid names for the species are *Navanax nyanyanus* (Edmunds 1968) – eastern Atlantic, *Navanax gemmatus* (Mörch 1863) – western Atlantic and *Navanax aenigmaticus* (Bergh 1893) – eastern Pacific.

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Introduction

Navanax is a small group of Aglajidae cephalaspideans with only two or three species currently recognized as valid. Aglajids are carnivorous sea slugs, generally lacking radula that feed by ingesting whole prey. Most of the 60 described species of Aglajidae inhabit shallow water tropical, subtropical and temperate regions. Contrary to most cephalaspideans, the shell of aglajids is vestigial and internal. Because most aglajids lack a radula, identification of species and higher taxa is often based on external morphological traits (including colour), reproductive anatomy and to some extent on the morphology of the internal shell. Authors have disagreed on the value of certain traits for classification and phylogenetic reconstruction (Rudman

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1978; Gosliner 1980), and a recent study has shown that colour might not be a good diagnostic feature at the species level (Ornelas-Gatdula *et al.* 2011).

The name *Navanax aenigmaticus* (Bergh 1893) has been used for a group of Aglajidae found in tropical and subtropical shallow water habitats in the eastern Atlantic, the western Atlantic and the eastern Pacific. Externally, specimens assigned to *Navanax aenigmaticus* are characterized by having light, elongate bodies with a series of longitudinal dark markings and iridescent blue or greenish spots along the parapodial margins (Fig. 1). The diet of all these organisms consists of other species of opisthobranchs, including cephalaspideans and other sea slugs.

One of the most puzzling aspects of the biology of the opisthobranchs assigned to *Navanax aenigmaticus* is that, if they are regarded as members of the same species, then *N. aenigmaticus* would have a disjunct geographical range. However, some external differences between specimens from the eastern Atlantic, western Atlantic and eastern Pacific could suggest that they are different species. Whereas eastern Atlantic specimens usually have a vivid colouration with yellow markings along the parapodial edges (Schrödl *et al.* 2007; Rudman 2007a), western Atlantic specimens may lack such yellow spots (Rudman 2000). Atlantic specimens have longitudinal lines, whereas Chilean specimens do not (Schrödl 2007a), and other eastern Pacific specimens also may have mottled rather than striped bodies (Rudman 2007b). Beyond these general differences, it is difficult to find consistent traits to distinguish between populations, and the external colouration is highly variable even among specimens from the same locality (Fig. 1).

Several species names have been introduced over the years for these three different populations or colour forms within each population. However, Gosliner (1980) and Zamora Silva (2008) revised material from all these

Fig. 1 Live photographs of some of the specimens sequenced including collection number and locality.

regions and found no consistent morphological differences, thus concluding that they are all members of the same species. Another main problem dealing with the taxonomy of this group is the availability of old names based on descriptions difficult to interpret using modern standards or providing confusing statements on type localities or names (see Schrödl 2007b).

In this paper, we will attempt to clarify the taxonomic status of these three populations using sequence data from two mitochondrial (COI and 16S) and a nuclear (H3) gene of specimens covering the entire range of this species. Additionally, some morphological traits (shell shape and external colouration) will be re-examined to supplement the detailed reproductive anatomy studies conducted by Gosliner (1980) and Zamora Silva (2008). Because of the confusing taxonomy of the group, we will refer to the three populations together as the '*Navanax aenigmaticus*' species complex. This paper also attempts to provide a review of the pertinent literature and type specimens to determine valid names for members of the species complex that best contribute to nomenclatural stability.

Because of the large, disjunct range of this species complex in isolated or potentially isolated regions, this paper has implications for understanding the biogeographical history of marine organisms across the Atlantic Ocean, the Isthmus of Panama and along the eastern Pacific and the western Atlantic. Another important aspect of this paper is to provide insights into divergence patterns of morphologically similar populations and potentially provide evidence of new cryptic diversity in the Atlantic Ocean.

Material and methods

Source of specimens

Fifty-five specimens covering the most of the geographical range of the '*Navanax aenigmaticus*' species complex were sequenced along with two outgroup taxa (Table 1). Specimens were collected by the authors, donated by colleagues, or obtained from museum collections. Specimens collected by the authors were photographed alive, narcotized using a 1 M solution of $MgCl_2$ and preserved in 70% or 100% EtOH. All the specimens are deposited at the following institutions: Natural History Museum of Los Angeles County (LACM), Zoologische Staatssammlung München, Germany (ZSM), Museo de Zoología, Universidad de Costa Rica (MZUCR), and the Department of Invertebrate Zoology and Geology, California Academy of Sciences, San Francisco (CASIZ).

Morphological examination

Eighteen specimens, representing all three geographical regions examined, were dissected (Table 1). The shell of each specimen was dissected and the surrounding tissue

removed manually. Only the calcified portion of the shell remained after treatment. Shells were rinsed in water, dried, mounted and sputter coated for examination with a scanning electron microscope (SEM) Hitachi S-3000N at the Natural History Museum of Los Angeles County.

Specimens and DNA extraction

DNA extraction was performed using either a hot Chelex® protocol or the DNeasy Blood and Tissue Kit (Qiagen). Approximately 1–3 mg of the foot or mantle was cut into fine pieces for extraction for both protocols. For the Chelex® extraction, the foot tissue was rinsed and rehydrated using 1.0 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min. A 10% (w/v) Chelex® 100 (100–200 mesh, sodium form; Bio-Rad, Hercules, CA, USA) was prepared using TE buffer. After rehydration, the mixture was then centrifuged, 975.00 μL of the supernatant was removed, and 175.00 μL of the Chelex® solution was added. Samples were then heated in a 56 °C water bath for 20 min and again heated in a 100 °C heating block for 8 min, and the supernatant was used for PCR. The DNeasy protocol supplied by the manufacturer was followed, with some modifications. The elution step was modified such that the first elution was collected using 100.00 μL of Buffer AE and was allowed to incubate at room temperature for 5 min. In a new test tube, a second elution step was conducted using 200.00 μL of Buffer AE and was also allowed to incubate at room temperature for 5 min. The first elution, or in some cases the second elution, was used for PCR.

Primer design

Colgan's universal H3 primers (Colgan *et al.* 1998) were used with all specimens to amplify the region of interest. Palumbi's universal 16S primers (Palumbi 1996) and Folmer's universal COI primers (Folmer *et al.* 1994) were used to amplify the regions of interest for all specimens except for the Cape Verde specimen. Internal primers specific for *Navanax* were designed for both 16S and COI for use with the Cape Verde specimen and were used with the complimentary universal primers for the gene (Table 2). The universal primers for each gene were also used with the outgroups *Navanax inermis* (Cooper, 1863) and *Chelidonura berolina* Marcus & Marcus 1970.

PCR amplification and sequencing

For most reactions, the master mix was prepared in a 50 μL volume using 34.75 μL H_2O, 5.00 μL Buffer B (ExACTGene; Fisher Scientific, Hampton, New Hampshire, USA), 5.00 μL 25 mM $MgCl_2$, 1.00 μL 40 mM dNTPs, 1.00 μL 10 mM primer 1, 1.00 μL primer 2, 0.25 μL 5 mg/mL Taq and 2.00 μL extracted DNA. Other reactions were carried out with the master mix prepared in

Table 1 List of specimens used in the study; including locality, voucher number and GenBank Accession numbers

| Species | Locality | Voucher no. | GenBank accession no. | | |
			H3	16S	COI
Chelidonura berolina	Bahamas	LACM 176429	HQ011892	HQ011857	HQ011868
Navanax inermis	Long Beach, California, USA	LACM 176388	JN402119	JN402154	JN402045
N. nyanyanus*	Ilha Boavista, Cape Verde	LACM 153125	JN402090	JN402138	JN402066
N. nyanyanus	Miamia, Ghana	ZSM Mol-20070253	JN402074	JN402123	JN402038
N. nyanyanus	Miamia, Ghana	ZSM Mol-20070241	JN402072	JN402121	JN402040
N. gemmatus	Bahamas	CASIZ 087317	JN402102	–	–
N. gemmatus*	Peanut Island, Florida, USA	LACM 176389	JN402105	JN402153	JN402056
N. gemmatus*	St. Anne's Bay, Jamaica	LACM 173262	JN402106	JN402137	JN402055
N. gemmatus*	St. Anne's Bay, Jamaica	LACM 173263	JN402089	JN402136	JN402067
N. gemmatus*	Curaçao, Netherlands Antilles	LACM 2004-94.8	JN402096	–	–
N. gemmatus	Limón, Costa Rica	–	JN402118	–	–
N. gemmatus	Pta Cahuita, Limón, Costa Rica	MZUCR 6925	JN402109	–	JN402048
N. gemmatus	Pta Cahuita, Limón, Costa Rica	MZUCR 6926	JN402110	JN402155	JN402049
N. gemmatus	Pta Cahuita, Limón, Costa Rica	–	JN402108	JN402156	JN402047
N. gemmatus	Pta Cahuita, Limón, Costa Rica	CASIZ 175767	JN402107	JN402151	JN402046
N. gemmatus	Pernambuco, Brazil	ZSM Mol-20090614	JN402086	JN402125	JN402026
N. gemmatus	São Paulo, Brazil	ZSM Mol-20100506	JN402069	JN402130	JN402043
N. gemmatus	São Paulo, Brazil	ZSM Mol-20100505	JN402070	JN402124	JN402042
N. aenigmaticus*	Bahía Magdalena, Baja California, Mexico	LACM 50-35.19	JN402098	–	–
N. aenigmaticus*	Rocas Alijos, Baja California Sur, Mexico	LACM 176390	JN402093	JN402140	JN402063
N. aenigmaticus*	Rocas Alijos, Baja California Sur, Mexico	LACM 176390	JN402094	JN402141	JN402062
N. aenigmaticus*	Rocas Alijos, Baja California Sur, Mexico	LACM 176390	JN402095	JN402142	JN402061
N. aenigmaticus*	Manzanillo, Colima, Mexico	LACM 176391	JN402116	JN402143	JN402060
N. aenigmaticus*	La Audiencia, Colima, Mexico	LACM 176392	JN402117	JN402144	JN402059
N. aenigmaticus*	Bahía Banderas, Jalisco, Mexico	LACM 71-83.51	JN402097	–	–
N. aenigmaticus*	Islas Marietas, Jalisco, Mexico	LACM 176393	JN402099	JN402145	JN402058
N. aenigmaticus*	Islas Marietas, Jalisco, Mexico	LACM 176393	JN402100	JN402146	JN402057
N. aenigmaticus	San Juan del Sur, Nicaragua	CASIZ 066862	JN402104	–	–
N. aenigmaticus	Playa Sámara, Guanacaste, Costa Rica	MZUCR 6923	JN402114	JN402148	JN402053
N. aenigmaticus*	Isla del Caño, Puntarenas, Costa Rica	LACM 1972-68.44	JN402088	–	JN402065
N. aenigmaticus	Punta Uvita, Puntarenas, Costa Rica	MZUCR 6922	JN402113	JN402147	JN402052
N. aenigmaticus	Isla Uva, Puntarenas, Costa Rica	CASIZ 175758	JN402111	JN402152	JN402050
N. aenigmaticus	Isla Uva, Puntarenas, Costa Rica	–	JN402112	JN402149	JN402051
N. aenigmaticus	El Tómbolo, Puntarenas, Costa Rica	MZUCR 6927	JN402115	JN402150	JN402054
N. aenigmaticus	Isla de Cocos, Costa Rica	CASIZ 073370	JN402103	–	–
N. aenigmaticus*	Islas de las Perlas, Panama	LACM 153502	JN402091	–	–
N. aenigmaticus*	Canal de Jicarón, Veraguas, Panama	LACM 153304	JN402092	JN402139	JN402064
N. aenigmaticus*	Isla Santa Cruz, Galapagos, Ecuador	LACM 34-189.10	JN402101	–	–
N. aenigmaticus	Bayovar, Peru	ZSM Mol-20100773	JN402076	JN402131	JN402037
N. aenigmaticus	Mancora, Peru	ZSM Mol-20100753	JN402087	JN402133	JN402027
N. aenigmaticus	Mancora, Peru	ZSM Mol-20091101	JN402085	–	JN402028
N. aenigmaticus	Mancora, Peru	ZSM Mol-20100754	JN402077	–	–
N. aenigmaticus	Mancora, Peru	ZSM Mol-20110019	JN402084	JN402126	JN402029
N. aenigmaticus	Mancora, Peru	ZSM Mol-20110046	JN402080	JN402129	JN402032
N. aenigmaticus	Mancora, Peru	ZSM Mol-20110020	–	JN402134	JN402033
N. aenigmaticus	Mancora, Peru	ZSM Mol-20110045	JN402075	–	–
N. aenigmaticus	Laguna Grande, Peru	ZSM Mol-20110047	JN402083	–	JN402030
N. aenigmaticus	Laguna Grande, Peru	ZSM Mol-20100035	–	JN402128	JN402035
N. aenigmaticus	Laguna Grande, Peru	ZSM Mol-20110049	JN402078	JN402120	JN402036
N. aenigmaticus	Laguna Grande, Peru	ZSM Mol-20110048	JN402071	JN402135	JN402041
N. aenigmaticus	Laguna Grande, Peru	ZSM Mol-20100777	JN402068	–	JN402044
N. aenigmaticus	Juan López, Chile	ZSM Mol-20110051	JN402082	JN402127	JN402031
N. aenigmaticus	Juan López, Chile	ZSM Mol-20110050	JN402081	–	–
N. aenigmaticus	Juan López, Chile	ZSM Mol-20100037	JN402079	JN402122	JN402034
N. aenigmaticus	Juan López, Chile	ZSM Mol-20100036	JN402073	JN402132	JN402039

*Specimens in which the shell was examined.

Table 2 Forward (F) and reverse (R) PCR primers used to amplify regions of the nuclear H3 gene and mitochondrial 16S and COI genes

Name	Sequence 5'–3'	Source
H3		
HexAF (F)	ATG GCT CGT ACC AAG CAG ACG GC	Colgan *et al.* (1998)
HexAR (R)	ATA TCC TTG GGC ATG ATG GTG AC	Colgan *et al.* (1998)
16S rRNA		
16Sar-L (F)	CGC CTG TTT ATC AAA AAC AT	Palumbi (1996)
16Sbr-H (R)	CCG GTC TGA ACT CAG ATC ACG T	Palumbi (1996)
16Sar-FAP (F)	AAA GAC GAG AAG ACC CTT AGA GTT TT	
16Sbr-FAP (R)	AAA ACT CTA GGG TCT TCT CG TCT TT	
COI		
LCO1490 (F)	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.* (1994)
HCO2198 (R)	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)
LCO-EIC (F)	ACA TCT TGC TGG TAT GTC TTC TAT TTT	
HCO-EIC (R)	AAA TAG AAG ACA TAC CAG CAA GAT GT	

a 25 μL volume using 1.00 μL 10× PCR buffer, 0.20 μL 10 mM dNTPs, 1.50 μL 25 mM MgCl$_2$, 0.025 μL (1.25 units/μL) Taq-Apex, 0.20 μL 25 μM primer 1, 0.20 μL 25 μM primer 2 and 1.00–2.00 μL extracted DNA. In some instances, 1.00–2.00 μL per sample of bovine serum albumen was added to this master mix. Reaction conditions for most of H3 and all of 16S were as follows: an initial denaturation for 2 min at 94 °C; 30 cycles of (i) denaturation for 30 s at 94 °C, (ii) annealing for 30 s at 50 °C and (iii) elongation for 1 min at 72 °C; and a final elongation for 7 min at 72 °C. Reactions for the remaining H3 were carried out using the following conditions: an initial denaturing step for 3 min at 94 °C; 2 min at 50 °C; 2 min at 72 °C; 35 cycles of (i) denaturation for 35 s at 94 °C, (ii) annealing for 1 min at 50 °C and (iii) elongation for 75 s at 72 °C (Latiolais *et al.*, 2006). Reaction conditions for COI an initial denaturation for 3 min at 95 °C, 35 cycles of (i) denaturation for 45 s at 94 °C, (ii) annealing for 45 s at 45 °C and (iii) elongation for 2 min at 72 °C, and a final elongation for 10 min at 72 °C.

PCR products yielding bands of appropriate size (approximately 375 bp for H3, 475 bp for 16S rRNA and 700 bp for COI) were purified using the Montage PCR Cleanup Kit (Millipore Billerica, MA, USA) or by using the reaction kit ExoSAP-IT (USB Scientific, Cleveland, OH, USA). The Cape Verde specimen yielded two fragments approximately 250 bp in length for 16S (16Sar-L + 16Sbr-FAP and 16Sar-FAP + 16S br-H), as well as two fragments approximately 475 and 300 bp in length for COI (LCO1490 + HCO-EIC and LCO-EIC + HCO2198, respectively).

Most of the cleaned PCR samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Each primer was diluted to 2.0 pmol/μL to send out for sequencing with the PCR products. PCR products were diluted to 6.0, 7.5 and 11.5 ng/μL for H3, 16S rRNA and COI, respectively. For the Cape Verde specimen, the fragments for 16S rRNA and COI were all diluted to 6.0 ng/μL, except for the 475-bp fragment (COI) that was diluted to 7.5 ng/μL. Samples were sequenced at the City of Hope DNA Sequencing Laboratory (Duarte, CA, USA) using chemistry types BigDye V1.1 for fragments <500 bp and BigDye V3.1 for fragments larger than 500 bp. For some samples from Costa Rica and Jamaica, DNA sequences were obtained using the 3.1 ABI Prism BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA, USA) in 10.00 μL reactions. Each reaction contained 0.50–2.00 μL of cleaned PCR product, 1.63 μL 5× reaction buffer, 0.50 μL 10 mM primer, 0.75 μL Big Dye (3.1 ABI) and water to 10.00 μL. These reactions were run on a BioRad MyCycler™ Thermocycler (software version 1.065; Bio-Rad Laboratories). Subsequently, DNA was precipitated by adding 2.50 μL EDTA (di-Na, pH 8.0) and sequential washing and pelleting in an Eppendorf centrifuge (Model 5810R) with 100% and then 70% EtOH. The pelleted DNA was denatured for 2 min at room temperature in 13.00 μL HiDi deionized formamide (Applied Biosystems, Inc.). The denatured, labelled DNA fragments were sequenced in both directions on an automated Genetic Analyzer (ABI-Prism 3100 and 3130XL; Applied Biosystems, Inc.) at the Center for Comparative Genomics at the California Academy of Sciences (San Francisco, CA, USA) according to the manufacture's instructions.

Phylogenetic analyses

Sequences for each gene were assembled and edited using either Geneious Pro 4.7.4 (Biomatters Ltd., Auckland, New Zealand) or Sequencher 4.7 (GeneCodes, Ann Arbor, MI, USA). Geneious was also used to extract the consensus sequence and to construct the alignment for each gene using the default parameters. The sequences were not trimmed after alignment. A total of 328 bp for H3, 411 bp for 16S and 658 bp for COI were used for the phylogenetic analyses. An analysis was conducted for each individual gene, as well as a combined analysis using all three genes concatenated. As indicated in Table 3, only taxa for which all three genes were successfully amplified were used in the combined analysis. To assess whether H3, 16S and COI have significantly conflicting signals, the incongruence length difference (ILD) test (Mickevich & Farris 1981; Farris *et al.* 1994), implemented in PAUP*4.0 as the partition homogeneity test (Swofford 2002), was conducted for all genes combined.

The levels of saturation for each gene and for the first and second vs. third codon positions of COI and H3 were

Table 3 Summary of each data set used for analysis with the best-fit evolutionary models and estimated parameters

Parameters	H3	16S	COI	H3 + 16S + COI
No. of specimens used in the study	53	38	43	36
No. of included characters	328	415	658	1401
Best-fit model	TrN	HKY+G	HKY+I+G	HKY+I
Frequency A	0.2329	0.3204	0.2596	0.2593
Frequency C	0.3338	0.1525	0.1645	0.1669
Frequency G	0.2653	0.2168	0.1563	0.1576
Frequency T	0.1680	0.3103	0.4197	0.4162
R-matrix [A-C]	1.0000	–	–	–
R-matrix [A-G]	1.1969	–	–	–
R-matrix [A-T]	1.0000	–	–	–
R-matrix [C-G]	1.0000	–	–	–
R-matrix [C-T]	3.7967	–	–	–
R-matrix [G-T]	1.0000	–	–	–
Γ shape (G)	–	0.2704	2.9806	–
Proportion of Invariant Sites (I)	–	–	0.6847	0.6904

investigated using the substitution saturation test developed by Xia et al. (2003) and Xia & Lemey (2009) implemented in the program DAMBE (Xia & Xie 2001).

The Akaike information criterion (Akaike 1974) was executed in jModelTest (Posada 2008) and MrModeltest v2.3 (Nylander 2004), to determine the best-fit model of evolution (Table 3).

Maximum likelihood analyses were conducted using PAUP*4.0 (Swofford 2002). Robustness of each clade was assessed by bootstrap support (Felsenstein 1985) based on 2000 replicates with heuristic search, TBR branch-swapping algorithm, multrees option and 100 random additions.

Bayesian analyses were executed in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), partitioned by gene (unlinked). The Markov chain Monte Carlo analysis was run with two runs of six chains for five million generations, with sampling every 100 generations. The default 25% burn-in was applied before constructing majority-rule consensus tree/s. Diagnostic nucleotides for each clade were identified visually in the alignments after collapsing identical haplotypes using the program COLLAPSE 1.2 (Posada 2004).

Population genetics analyses

The population structure for the COI and H3 haplotypes was analysed by performing a two-level hierarchical analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.512 (Excoffier & Lischer 2010). Samples were allocated to populations according to their geographical distribution and position in the phylogenetic tree. The following three populations were defined: 'Eastern Pacific', 'Western Atlantic', and 'Eastern Atlantic'. F-statistic analogues, designated Φ-statistics, based on Tamura-Nei corrected sequence divergences were calculated among haplotypes and haplotype frequencies. A non-parametric permutation procedure was used to test whether statistics were significantly different from zero.

Results

Morphology

Examination of live specimens and photographs from collectors as well as field data has revealed some differences between members of the 'Navanax aenigmaticus' species complex. For example, adult specimens from the western Atlantic (maximum size 60 mm long, but commonly around 30 mm long) are often smaller and less colourful than eastern Pacific (maximum size 75 mm long) and eastern Atlantic animals (maximum size 50 mm long). Additionally, the blue spots on the margin of the parapodia of western Atlantic animals are generally smaller than those of the two other populations. Some eastern Pacific animals often have yellow spots all over the body, rather than yellow lines, but others do not. Eastern Atlantic specimens usually have a vivid colouration with yellow markings along the parapodial edges. These differences are not sufficiently consistent to clearly separate the three populations and could also be influenced by environmental factors.

Scanning electron microscope examination of the shell morphology of members of the 'N. aenigmaticus' species complex has revealed several differences between specimens from different regions and of different developmental stages (Fig. 2). Three juvenile specimens examined possess a fully calcified shell with a short apical wing and the protoconch is partially visible. In adult specimens, only the apical rim of the shell is calcified, the wing is fully developed and the protoconch is completely covered by calcified shell growth. Adult animals from the Eastern Pacific have a large, elongate protoconch calcified region, about 1 mm long, sitting almost at right angles to the large recurved calcified apical wing. In adult animals from the Western Atlantic, the protoconch calcified region is substantially smaller, about 250 μm, but the apical region of the shell extends considerably opposite to the wing, so the protoconch is centrally located in the shell. In Eastern Atlantic specimens, the protoconch calcified region is about 1 mm long but much wider than in the Eastern Pacific specimens, the wing is also shorter and the shell expands slightly opposite to the wing, so the protoconch is more centrally located. As in the case of the external morphology, these differences are too unreliable to base taxonomic conclusions on them alone; however, differences in protoconch size could be reliable indicators of different
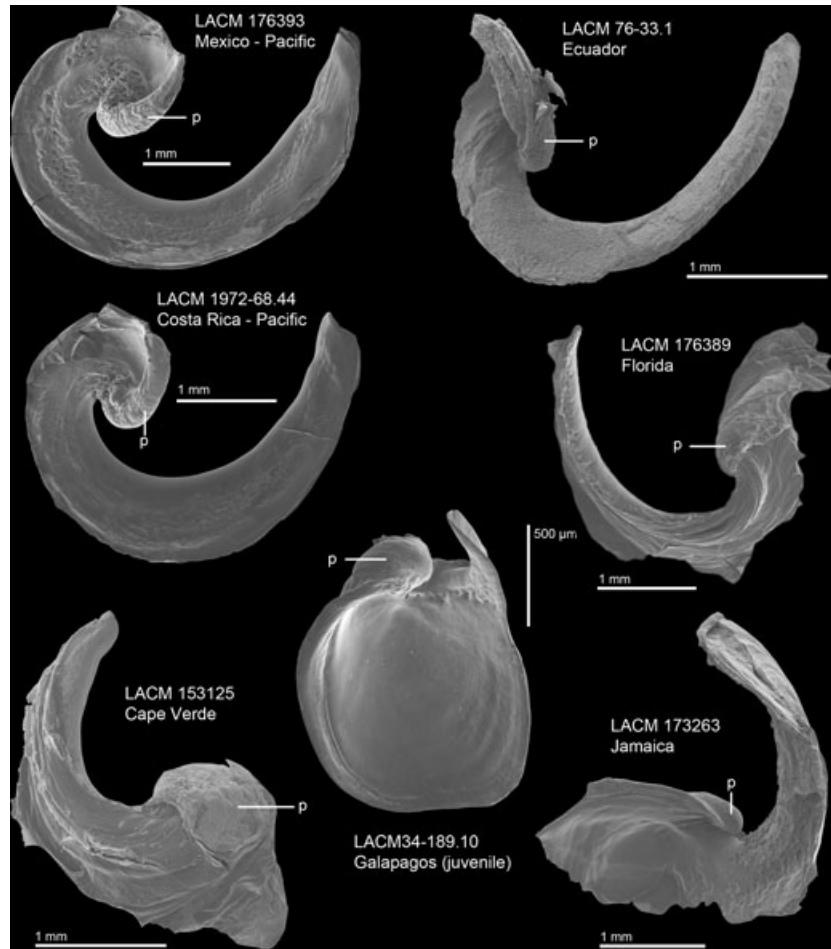
Fig. 2 Scanning electron micrographs of the shells of seven specimens. Museum collection number, locality and scale bar indicated for each specimen. Abbreviations: p = calcified protoconch region.

developmental modes and could potentially be useful to separate species. The reproductive anatomy of all three populations was examined by Gosliner (1980) and Zamora Silva (2008) who found no consistent differences between the three populations.

Sequence analyses

The H3 gene was the most conserved among all taxa, but some variation was observed. The three main clades produced by the phylogenetic analyses are supported by two molecular synapomorphies each. All Western Atlantic specimens have a G in position 160 of the alignment, whereas both Eastern Atlantic and Eastern Pacific have a C. In position 313, all Eastern Atlantic animals have a T, whereas both Western Atlantic and Eastern Pacific have a C. Together, all members of the '*N. aenigmaticus*' species complex are supported as a monophyletic group by six synapomorphies in H3 not present in *N. inermis* or *C. berolina* in positions 40 (G), 136 (T), 139 (G), 268 (A), 283 (C), 307 (C).

The 16S rRNA gene (ingroup pairwise identity 98.5%) was more variable than the H3 gene (ingroup pairwise identity 99.8%), and the COI gene (ingroup pairwise identity 94.5%) was the most variable of all three genes. For COI, five of 23 specimens yielded sequences that were 13 bp shorter at the 3′ end, but the longer sequences were not manually truncated at the 3′ end to match the shorter sequences as the 13 bp contained phylogenetic information. In both 16S and COI alignments, each of the members of the '*N. aenigmaticus*' species complex are supported by numerous synapomorphies (11 for 16S and 50 for COI).

The saturation analyses showed insignificant levels of saturation for all three genes (COI: lss<lss.c, $P = 0.000$; 16S: lss<lss.c, $P = 0.000$; H3: lss<lss.c, $P = 0.000$) even when the third codon positions of COI and H3 were analysed independently. The ILD test showed no significant conflicting signals between the three genes combined ($P = 0.997$).

Phylogenetic analyses

The combined analysis of the three genes H3, 16S and COI produced maximum likelihood and Bayesian consensus trees very similar. Only the Bayesian tree is illustrated

(Fig. 3) including both posterior probabilities and bootstrap values for each clade. In this consensus tree, members of the 'Navanax aenigmaticus' species complex form a monophyletic group with strong support (posterior probability = 1.00; bootstrap = 80). Within the 'N. aenigmaticus' species complex, three main clades were recovered, all of them well supported. The three clades form an unresolved polytomy at the base. One of the clades contains all specimens examined from the Eastern Atlantic, including Cape Verde and Ghana (type locality of Chelidonura nyanyana Edmunds 1968). The support for this clade is strong (posterior probability = 1.00; bootstrap = 96). Within this clade, the Ghana specimens cluster together but this is weakly supported (posterior probability = 0.69; bootstrap = 69). Another clade includes all specimens examined from the Western Atlantic, including Jamaica, Florida, Costa Rica and Brazil (posterior probability = 1.00; bootstrap = 100). Although the relationships among members

of this clade are largely unresolved because of lack of structure and support in the tree, specimens from Brazil are basal to those from the Caribbean and Florida, and all Caribbean and Florida specimens are clustered in a single clade supported in the Bayesian analysis (posterior probability = 0.81), but not in the maximum likelihood analysis. The Brazilian specimens do not form a monophyletic group, but a basal grade. The last main clade in the consensus tree includes specimens from the Eastern Pacific, including Mexico, Costa Rica, Panama, Peru and Chile. The overall support for the Eastern Pacific clade is strong (posterior probability = 1.00; bootstrap = 94). As in the case of the Western Atlantic, the relationships among members of this clade are unresolved, but specimens from South America tend to be more basal. A weakly supported clade in the Bayesian analysis (posterior probability = 0.72) includes all specimens from the Central and North America, along with one South American specimen from Chile.
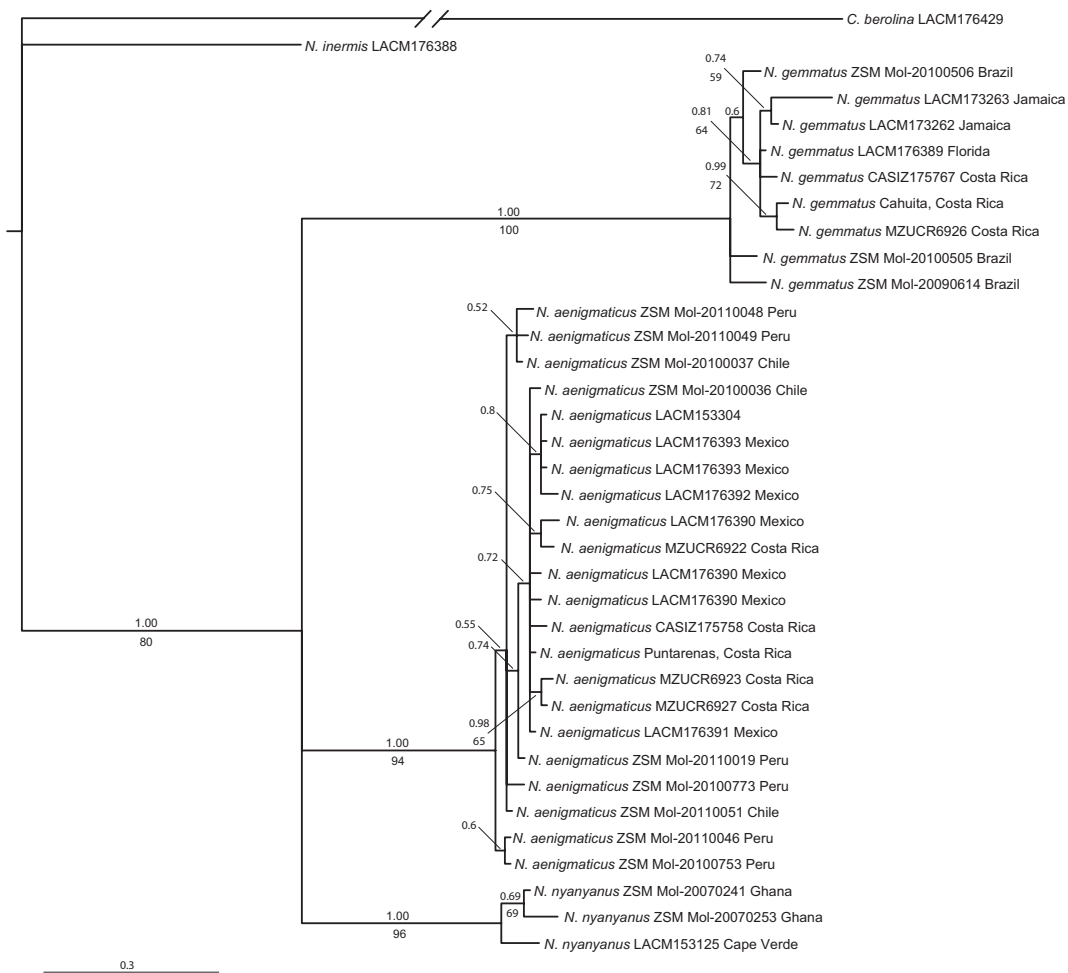

Fig. 3 Bayesian tree of concatenated dataset (COI, 16S, H3). Support values for likelihood and Bayesian approaches are shown only for nodes with bootstrap values over 50 or posterior probability over 0.5.

When the H3 gene is analysed alone, the trees produced are largely unresolved. Only two clades are supported, the Eastern Atlantic clade (posterior probability = 0.91; bootstrap = 64) and the Western Atlantic clade (posterior probability = 0.92; bootstrap = 65); the monophyly of the 'N. aenigmaticus' species complex is weakly supported in the maximum likelihood tree (bootstrap = 62). Results from the analysis of the 16S gene alone are similar, and only two clades within the 'N. aenigmaticus' species complex are well supported, the Eastern Atlantic clade (posterior probability = 1.00; bootstrap = 78) and the Western Atlantic clade (posterior probability = 1.00; bootstrap = 94). However, in this analysis, N. aenigmaticus is not monophyletic and N. inermis is sister to the Eastern Atlantic and Western Atlantic clades of N. aenigmaticus. The support for this clade is relatively strong (posterior probability = 0.99; bootstrap = 79). Finally, the analysis of the COI gene alone recovered the three main clades present in the three-gene analysis, Eastern Atlantic (posterior probability = 1.00; bootstrap = 100), Western Atlantic (posterior probability = 0.99; bootstrap = 94) and Eastern Pacific (posterior probability = 0.95; bootstrap = 89). The 'N. aenigmaticus' species complex is also monophyletic (posterior probability = 0.95; bootstrap = 89).

Population genetics analyses

For the AMOVA analysis of CO1 haplotypes, among-population differentiation explained 95.42% of the covariance component, while within-population differentiation explained 4.58% (Φ_{ST} = 0.95423, P = 0.0000, based on 10 100 permutations). For the AMOVA analysis of H3 haplotypes, among-population differentiation explained 89.68% of the covariance component, while within-population differentiation explained 10.32% (Φ_{ST} = 0.89683, P = 0.0000, based on 10 100 permutations). The pairwise Φ_{ST} values for both genes are summarized in Table 4 all population comparison values were significantly higher than zero.

Table 4 Matrix of Pairwise Φ_{ST} values between populations for CO1 (lower triangular) and H3 (upper triangular), including P values. All values are significantly above 0

	Eastern Pacific	Eastern Atlantic	Western Atlantic
Eastern Pacific	–	0.90105, P = 0.000	0.88700, P = 0.000
Eastern Atlantic	0.93971, P = 0.001	–	0.93343, P = 0.0019
Western Atlantic	0.95897, P = 0.000	0.94650, P = 0.000	–

Discussion

Systematics and classification

Gosliner (1980) and more recently Zamora Silva (2008) examined the reproductive anatomy, shell morphology and other characteristics of Caribbean and Eastern Pacific populations of 'Navanax aenigmaticus'. Both authors concluded that anatomically these two populations are indistinguishable and therefore belong to the same species. Whereas Gosliner (1980) concluded that the oldest valid name for the species should be Navanax aenigmaticus (Bergh 1893), Zamora Silva (2008) suggests the possibility that Posterobranchaea maculata d'Orbigny, 1837 might have priority. In this study, we found some diverging trends rather than diagnostic differences in body size and colour pattern between Pacific and Atlantic specimens. There are also some minor morphological differences in the shell of specimens from the three different regions that appear to be consistent in all the specimens examined (see results section). However, these differences are not clear enough to contradict previous studies (Gosliner 1980; Zamora Silva 2008). Thus, the morphological studies are inconclusive.

On the contrary, molecular evidence shows well-supported divergences between the eastern Atlantic, western Atlantic and eastern Pacific populations of the 'N. aenigmaticus' species complex. Thus, we consider individuals from these three regions to belong to different cryptic species. All these three species are supported by molecular synapomorphies in both nuclear and mitochondrial genes and significant molecular differences in the AMOVA tests.

An important problem is to determine the oldest available name for each of the three species recognized in this study. Some of the oldest names available are based on brief descriptions of the external morphology of live and preserved animals and could represent almost any aglajid. The critical question is whether we make an attempt to resurrect and fix older names (which in some cases might require designation of neotypes) or use a more pragmatic approach and use the names introduced with descriptions that we can recognize. We have chosen the latter.

According to the literature, the oldest available name for species of the eastern Atlantic 'Navanax aenigmaticus' clade is possibly Posterobranchus orbignyanus de Rochebrune 1881. The original publication (de Rochebrune 1881) included a brief description of the preserved animal from Santiago (Santiagu), Cape Verde (West Africa) and was followed up by the publication of a drawing of the species (de Rochebrune 1882). The type specimen, collected by Monsieur de Céssac, was deposited at the Muséum National d'Historie Naturelle, Paris, where it is no longer traceable (Valdés & Héros 1998). Gosliner (1980) considered that it is probable that P. orbignyanus is synonymous

with *Navanax aenigmaticus* but decided to consider *P. orbignyanus* as a *nomen oblitum*. At the time of Gosliner's (1980) paper, the status *nomen oblitum* did not exist (ICZN, 1999: Article 23.12). Espinosa & Ortea (2001) agreed with Gosliner (1980) in recognizing only one valid species in the 'Navanax aenigmaticus' species complex but considered that *P. orbignyanus* is the oldest available name for the species; thus, it has priority. We have re-examined the original description and original drawing of *P. orbignyanus* (de Rochebrune 1881, 1882) and have been unable to positively identify the species. However, the decision by Espinosa & Ortea (2001) to resurrect this name prevents us from regarding *P. orbignyanus* as a *nomen oblitum* (ICZN, 1999: Article 23.9.2). The oldest name we can recognize in the West Africa region is *Chelidonura nyanyana* Edmunds 1968; based on specimens from Ghana and described in detail including a drawing of the living animal (Edmunds 1968). Therefore, we have decided to use this name as valid for the eastern Atlantic species. The holotype of *Navanax nyanyanus* is deposited at the Natural History Museum, London (Edmunds 1968). *Chelidonura africana* Pruvot-Fol, 1953 is a distinct species (Martínez et al. 2002).

The oldest name for western Atlantic members of the 'Navanax aenigmaticus' clade is possibly *Doridium gemmatum* Mörch 1863; described from a single specimen from St. Thomas, Virgin Islands. Mörch (1863) described the animal as yellow with longitudinal black lines and green iridescent spots. Gosliner (1980) recognized that *D. gemmatum* is a synonym of *N. aenigmaticus* but regarded *D. gemmatum* as a *nomen oblitum*. Again, at the time of Gosliner's (1980) paper, the status *nomen oblitum* did not exist (ICZN, 1999: Article 23.12). Recently, Ortea et al. (2007) contradicted the previous decision to synonymize all species in the 'Navanax aenigmaticus' species complex and resurrected the name *D. gemmatum* as valid for the western Atlantic species, therefore preventing the designation of this name as *nomen oblitum*. The type material of *Doridium gemmatum* Mörch 1863 is not available at the Zoologisk Museum, Copenhagen; however, we can recognize the characteristics of this species based on the original description. No other Caribbean species of Aglajidae has a combination of longitudinal black lines and rows of iridescent green or blue spots. Bergh (1893) introduced the name *Doridium punctilucens* Bergh 1893 based on preserved specimens from St. Thomas and Guadeloupe (Caribbean), with a detailed description and a drawing of the shell. Gosliner (1980) regarded *D. punctilucens* as a synonym of *N. aenigmaticus* and considered the older name as *nomen oblitum*, retaining *N. aenigmaticus*. One of the syntypes of *D. punctilucens* (from Guadeloupe) is preserved in the collections of the Zoologisk

Museum, Copenhagen (GAS-2164). Examination of this specimen confirmed its identity as a member of the 'Navanax aenigmaticus' clade. Over half a century later, Marcus (1955) described *Chelidonura evelinae* Er. Marcus, 1955 from Brazil. The characteristics of this species are easily recognizable in the original description, and there is no question that this is a junior synonym of *D. gemmatum* as it was determined by Gosliner (1980). Marcus & Marcus (1970) described *Chelidonura evelinae dica* Marcus & Marcus, 1970 from Curaçao (Caribbean). Gosliner (1980) regarded this subspecies as a synonym of *N. aenigmaticus*. We agree with this decision and include this taxon in the western Atlantic species of the 'Navanax aenigmaticus' clade.

The oldest name for the eastern Pacific species is possibly *Posterobranchaea maculata* d'Orbigny, 1837 originally described from Valparaiso, Chile. Schrödl (2007b) discussed the original description of this species and was unable to determine its identity with certainty. We agree with this conclusion and therefore regard *P. maculata* as an uncertain species. The type material of *P. maculata* is not present at the Muséum National d'Historie Naturelle, Paris (Valdés & Héros 1998). Bergh (1893) introduced the name *Navarchus aenigmaticus* Bergh 1893 from the Gulf of Panama (Pacific Ocean) with a drawing of the penis and prostate and no description. In a second paper, Bergh (1894) provided a detailed description of *N. aenigmaticus*. This is the oldest recognizable description for eastern Pacific members of the 'Navanax aenigmaticus' species complex.

In conclusion, according to our interpretation of the literature, we propose the following list of valid species, synonyms and geographical range for members of the 'Navanax aenigmaticus' clade:

Eastern Atlantic
Navanax nyanyanus (Edmunds 1968). Geographical range: Cape Verde and Ghana.
 Synonyms:
 ?*Posterobranchus orbignyanus* de Rochebrune 1881: 265, pl. 18, fig. 5 (uncertain status)
 Chelidonura nyanyana Edmunds 1968: 83–85, fig. 1

Western Atlantic
Navanax gemmatus (Mörch 1863). Geographical range: From Florida to Southeastern Brazil.
 Synonyms:
 Doridium gemmatum Mörch 1863: 25–26
 Doridium punctilucens Bergh 1893: 131–133, pl. 8, fig. 16
 Chelidonura evelinae Marcus 1955: 95–101, figs 8–19
 Chelidonura evelinae dica Marcus & Marcus 1970: 14–15, figs 11–13

Eastern Pacific

Navanax aenigmaticus (Bergh 1893). Geographical range: From Southern California to Northern Chile.

Synonyms:

?*Posterobranchaea maculata* d'Orbigny, 1835–1843 [1837]: 203–204, pl. 17, figs 6–9 [1835] (uncertain status)

Navarchus aenigmaticus Bergh 1893: 134, pl. 8, fig. 5

In the absence of a comprehensive phylogeny of the Aglajidae, the validity of *Navanax* and its relationships with other aglajids remain unclear.

Biogeography

The scenario described here supports a strong biogeographical structure in the *Navanax aenigmaticus* species complex; however, because of the lack of resolution, it is impossible to determine the series of events that lead to the separation of the three species. The formation of the Isthmus of Panama about 3.1 Ma (Coates & Obando 1996) is the most likely event that splits the ranges of the western populations (Eastern Pacific and Western Atlantic), but the mechanism and timing of separation of Western Atlantic and Eastern Atlantic populations remains elusive.

As mentioned earlier, there is a certain level of biogeographical structure in both *Navanax gemmatus* (Western Atlantic) and *Navanax aenigmaticus* (Eastern Pacific). In both cases, South American specimens tend to be basal to North and Central American specimens. In the case of *N. gemmatus*, a clade containing only Caribbean and Florida specimens is well supported, but Brazilian animals do not form a monophyletic group, but rather an unresolved basal grade. In *N. aenigmaticus*, specimens from Peru and Chile are mainly basal but do not form a monophyletic group. Only one specimen from Chile is in the same clade as the Central and North American animals.

Although the available data does not provide a clear and well-resolved picture of the relationships among members of *N. gemmatus* and *N. aenigmaticus* it appears that there is a certain level of genetic isolation between north and south populations. It is intriguing that both *N. gemmatus* and *N. aenigmaticus* specimens from cooler areas, such as Brazil, and Chile and Peru, appear to be more basal in the phylogeny than specimens from tropical areas. This could be an artefact of lack of resolution in the analyses. More variable markers such as microsatellites or intron regions could be better alternatives to solve population structure questions within the two species.

Acknowledgements

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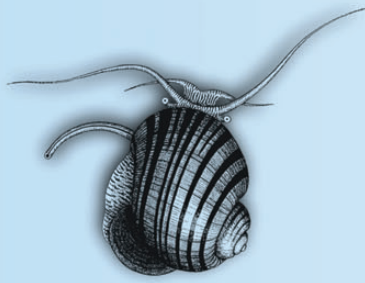
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3. RESULTS

<u>Chapter 7</u>

Padula V, Araújo AK, Matthews-Cascon H, Schrödl M (2014) Is the Mediterranean nudibranch *Cratena peregrina* (Gmelin, 1791) present in the Brazilian coast? Integrative species delimitation and description of *Cratena minor* n. sp. *Journal of Molluscan Studies* **80**: 575-584.

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Is the Mediterranean nudibranch *Cratena peregrina* (Gmelin, 1791) present on the Brazilian coast? Integrative species delimitation and description of *Cratena minor* n. sp.

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ABSTRACT

One of the main difficulties in the taxonomy of heterobranch sea slugs is the interpretation of small morphological and body colour differences in a group of specimens, sympatric or allopatric, as variation of a single species or indicative of similar, but different, species. The aeolid *Cratena peregrina* is one of the most common and typical nudibranchs from the Mediterranean Sea and was recently informally recorded from Senegal, South Africa, India and in the western Atlantic. In the present work, we investigate the potential presence of *C. peregrina* on the coast of Brazil. Brazilian and Mediterranean specimens are compared through multiple approaches, including (1) a molecular phylogenetic analysis based on a mitochondrial and a nuclear marker (cytochrome *c* oxidase subunit I and H3, respectively); (2) performing population analyses such as haplotype networks via TCS and Birky's coalescence-based K/θ ratio; (3) automatic barcode gap discovery and (4) comparative morphological study. As a result of our integrative species delimitation approach, we conclude that the morphological and body colour differences observed between Mediterranean and Brazilian specimens are not due to intraspecific variation in *C. peregrina* and that *C. peregrina* is not present in Brazil. Instead, Brazilian specimens belong to a new species, *C. minor* n. sp., which is described herein. We use this case study to discuss currently available methods of species delimitation and their integrative application to heterobranch sea slugs.

INTRODUCTION

For heterobranch sea slugs, specimens with the same or very similar external morphology and body colour pattern, generally from the same ocean region or basin, are traditionally regarded as conspecific (e.g. Schrödl, 2003; Valdés *et al.*, 2006). Internal morphology, such as of radula, jaws and reproductive system, usually complement the taxonomic study (Thompson & Brown, 1984). However, one of the main difficulties in the taxonomy of sea slugs is the interpretation of small morphological and body colour differences in a group of specimens, sympatric or allopatric. Do these differences represent variation of a single species or are they indicative of morphologically similar, but different, species? Recently, the addition of more detailed studies, the use of molecular tools and an integrative taxonomic approach have improved the capacity of taxonomists to delineate species and increased the discovery of previously unknown, mostly cryptic, species (e.g. Jörger *et al.*, 2012; Ornelas-Gatdula *et al.*, 2012; Krug *et al.*, 2013). These recent studies have also revealed that some taxonomic characters are not as informative as traditionally believed and have at the same time highlighted new, previously overlooked characters (e.g. Neusser, Jörger & Schrödl, 2011; Carmona *et al.*, 2013; Churchill *et al.*, 2013; Krug *et al.*, 2013).

Concerning nudibranchs, some recent studies using a molecular approach have focused on potential complexes of species, producing interesting results. Two forms, one with short and one with long cerata, of the aeolid *Flabellina verrucosa* were confirmed to be conspecific (Eriksson, Nygren & Sundberg, 2006). The

Table 1. List of specimens used for phylogenetic and species delimitation analyses.

Species	Locality	Voucher/source	GenBank accession number	
			COI	H3
Aeolidiella alderi	–	GenBank	HQ616766	HQ616795
Phidiana lynceus	–	GenBank	JX087562	JX087634
Learchis poica	–	GenBank	JQ699632	JQ699468
Sakuraeolis enosimensis	–	GenBank	HM162758	HM162591
Sakuraeolis enosimensis	–	GenBank	HQ010503	HQ010472
Cratena peregrina	France, Banyuls	ZSM Mol 20020957	KJ940481	KM079349
Cratena peregrina	France, Banyuls	ZSM Mol 20020957	–	KM079350
Cratena peregrina	Croatia, Crveni Otok	ZSM Mol 20100125	KJ940480	KM079347
Cratena peregrina	Croatia, Crveni Otok	ZSM Mol 20100125	–	KM079348
Cratena peregrina	Spain, Andalucia	ZSM Mol 20130772	KJ940482	KM079351
Cratena minor n. sp.	Brazil, Pernambuco, Itapessoca	ZSM Mol 20110345	KJ940476	KM079346
Cratena minor n. sp.	Brazil, Pernambuco, Itapessoca	ZSM Mol 20110338a	KJ940477	KM079341
Cratena minor n. sp.	Brazil, Pernambuco, Itapessoca	ZSM Mol 20110338b	KJ940478	KM079342
Cratena minor n. sp.	Brazil, Pernambuco, Itapessoca	ZSM Mol 20110338c	–	KM079343
Cratena minor n. sp.	Brazil, Pernambuco, Itapessoca	ZSM Mol 20110338d	–	KM079344
Cratena minor n. sp.	Brazil, Pernambuco, Itapessoca	MZSP 116702	KJ940479	KM079345

subspecies proposed for the dorid *Doriopsilla areolata* Bergh, 1880, by Valdés & Ortea (1997), were not recovered by molecular data (Goodheart & Valdés, 2013). In another case, molecular phylogenetic analyses indicated that the two sympatric, morphologically and ecologically distinct species *Dondice occidentalis* (Engel, 1825) and *D. parguerensis* Brandon & Cutress, 1985 were not reciprocally monophyletic (Gonzalez, Hanson & Valdés, 2013). In the broader phylogenetic work of Carmona *et al.* (2013), some morphologically identical or very similar aeolid specimens were recognized as belonging to different species based mostly on the divergence of cytochrome *c* oxidase subunit I (COI) and reciprocal monophyly. In some cases, the number of specimens was small, raising doubts if the conclusions could be influenced by a more comprehensive sampling or with additional lines of evidence (see De Salle, Egan & Sidall, 2005; Jörger *et al.*, 2012).

In this study we investigate the potential presence of the aeolid *Cratena peregrina* (Gmelin, 1791), one of the most common and typical nudibranchs from the Mediterranean Sea, on the coast of Brazil. This species has a very characteristic body colour pattern, with a whitish body, dark red to dark blue digestive gland branches in the cerata, an orange band on the rhinophores and a pair of rectangular orange spots on the head (Gmelin, 1791; Rudman, 1999). Recently, specimens with these characteristics have been photographed in other regions of the world, such as Senegal, South Africa, India and in the western Atlantic (Poddubetskaia, 2003; Valdés *et al.*, 2006; Debelius & Kuiter, 2007; Rudman, 2009), implying that *C. peregrina* could have a wide geographical distribution outside the Mediterranean Sea and surrounding areas. The first record in the western Atlantic was made by Valdés *et al.* (2006), as *C.* cf. *peregrina*, based on material photographed in Florida. More recently, Galvão Filho, Meirelles & Mathews-Cascon (2011) recorded *C.* cf. *peregrina* and egg masses from Ceará, northeastern Brazil.

Under the unified species concept (De Queiroz, 2007), we herein evaluate whether Mediterranean and Brazilian specimens are conspecific or not. Through an integrative taxonomic framework, we compare material from both regions through (1) molecular phylogenetic analyses based on a mitochondrial and a nuclear marker; (2) using population genetic approaches (e.g. K/θ ratio; Birky, 2013); (3) automatic barcode gap discovery (ABGD; Puillandre *et al.*, 2012) and (4) comparative

morphological study. Based on this case we discuss methods and concepts of integrative species delimitation approaches suitable for heterobranch sea slugs.

MATERIAL AND METHODS

Taxon sampling

Brazilian and Mediterranean specimens of *Cratena* were collected manually by the authors and colleagues through free and SCUBA diving. Specimens were photographed alive, narcotized using a 1 M solution of $MgCl_2$ and preserved in 70 or 96% EtOH. Material is deposited at Prof. Henry Ramos Matthews, series B, Malacological Collection of the Universidade Federal do Ceará (CMPHRM-B), Museu de Zoologia da Universidade de Sao Paulo (MZSP) and in the Zoologische Staatssammlung München (ZSM). We tried to obtain sequences of additional *Cratena* species, such as *C.* cf. *affinis* (Baba, 1949) and *C. lineata* (Eliot, 1905), but the attempts were not successful. *Cratena pilata* (Gould, 1870) sequences in GenBank were far distant from *C. peregrina* sequences in BLAST searches; therefore, and because they originated from unpublished works, they were not included in our final phylogenetic analysis. COI and H3 sequences of additional facelinid species *Sakuraeolis enosimensis*, *Learchis poica*, *Phidiana lynceus* and the aeolidiid *Aeolidiella alderi* were obtained from GenBank and included in the analysis (Table 1). *Aeolidiella alderi* was selected as outgroup.

DNA extraction, amplification and sequencing

Genomic DNA of each specimen was extracted from a small foot fragment using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co.), following the manufacturer's instructions. Two markers were amplified through polymerase chain reaction (PCR): COI (*c*. 655 bp) using the universal primers of Folmer *et al.* (1994) (LCO1490 5′-GGTCAACAAATCATAAAGA TATTGG-3′; HCO2198 5′-TAAACTTCAGGGTGACCAAA AATCA-3′) and nuclear histone H3 (*c*. 330 bp) using the primers of Colgan, Ponder & Eggler (2000) (H3aF 5′-ATGGC TCGTACCAAGCAGACVGC-3′; H3aR 5′-ATATCCTTRGG CATRATRGTGAC-3′). PCR amplification was performed in 25 ml reaction volume containing 22 ml of water, 0.5 ml of a

forward and reverse PCR primer (10 pm/μl), 2 ml of template DNA solution and one puReTaq Ready-To-Go PCR Bead (GE Healthcare). The cycling parameters for amplification consisted of an initial denaturation for 5 min at 94 °C, followed by 36 cycles of denaturation for 45 s at 94 °C, annealing for 50 s at 50 °C for both genes and extension for 200 s at 72 °C and ending with a final 10 min extension at 72 °C. Successful PCR products were purified using the NucleoSpin Extract II (Macherey-Nagel GmbH & Co.). Cycle sequencing using Big Dye 3.1 and the PCR primers (10 pm/μl) was conducted in the Genomic Service Unit of the Department of Biology, Ludwig-Maximilians-University Munich.

Sequence alignment and phylogenetic analyses

Sequences were edited using MEGA5 (Tamura *et al.*, 2011) and consensus sequences were generated in BioEdit (Hall, 1999). Alignments were generated with Muscle (Edgar, 2004) using the default settings. Testing the evolutionary models was carried out with Modeltest v. 3.7 (Posada & Crandall, 1998). Substitution saturation rate of H3 and COI were measured with Xia's method implemented in DAMBE v. 5.2.31 (Xia & Xie, 2001), for combined first and second codon positions, and for third codon position separately, using proportion of variation sites value of the best model obtained from Modeltest. The single-gene dataset was concatenated automatically using FASconCAT v. 1.0 (Kück & Meusemann, 2010). Maximum likelihood (ML) single-gene and gene trees of the concatenated dataset were generated using RaxML v. 7.2.6 (Stamatakis, 2006) and node support was assessed with nonparametric bootstrapping with 1,000 replicates. ML trees were visualized in FigTree v. 1.2 (http://tree.bio.ed.ac.uk/software/figtree/) and edited for publication in Corel Photo-Paint X6.

Species delimitation and network analyses

Diagnostic characters for COI were obtained through character attribute organization system (CAOS) software (Sarkar *et al.*, 2002; Sarkar, Planet & DeSalle, 2008; Bergmann *et al.*, 2009), including homogeneous and heterogeneous single pure character attributes (see Jörger & Schrödl, 2013), following the procedure described by Jörger & Schrödl (in press). Diagnostic characters for H3 were checked by eye. In both cases the nucleotide data alignments generated were used for the phylogenetic analysis. Position numbers of diagnostic characters refer to the position in the alignment, which can be accessed in the data matrices deposited in TreeBASE (www.treebase.org). ABGD (Puillandre *et al.*, 2012) and the K/θ method (Birky, 2013) were used in species delimitation analyses. ABGD is independent of predefined species entities and was applied to both COI and H3 datasets including *Cratena peregrina*, the Brazilian *Cratena* and their most closely related species in the phylogeny presented herein (*Sakuraeolis enosimensis*). The K/θ ratio method measures the sequence difference between putative species (e.g. well supported clades on single gene trees) and compares it with differences within species. It was applied for the COI dataset, comparing *C. peregrina* and the Brazilian *Cratena*. Uncorrected mean p-distances between COI sequences among each *Cratena* clade for calculation of θ and uncorrected and corrected (Kimura-2 parameter) mean COI p-distances between the two *Cratena* clades for calculation of K were obtained in MEGA5 (Tamura *et al.*, 2011). Minimum and maximum pairwise uncorrected p-distances of COI within and between clades/species were calculated with Species Identifier (Meier *et al.*, 2006). Haplotype networks for COI were constructed using statistical parsimony (Templeton, Crandall & Sing, 1992), implemented in the program TCS v. 1.21 (Clement, Posada & Crandall, 2000) with a connection limit of 95%.

Morphology

To check if there is any correspondence between the results of our molecular phylogen and species delimitation analyses and morphology, five specimens from two Brazilian localities (Ceará and Pernambuco) and four specimens from three localities in the Mediterranean (Spain, France and Croatia) were studied externally and internally. Morphological data on *C. peregrina* available in databases, such as Sea Slug Forum (www.seaslugforum.net) and Nudi Pixel (www.nudipixel.net), were also considered. For the study of the radula, jaws and reproductive system, specimens were dissected under a stereomicroscope. The buccal bulb was manually cleaned and immersed in a solution of 10% sodium hydroxide to dissolve soft tissues. Cleaned jaws and radula were transferred to distilled water and mounted for photography in the scanning electronic microscope LEO 1430VP, at the ZSM. For the study of the reproductive system, it was first cleaned from adjacent systems and then extracted from the body cavity and drawn, using a camera lucida.

RESULTS

Molecular data

The saturation analyses showed insignificant levels of saturation, even when the third codon positions of COI and H3 were analysed independently. The combined dataset yielded a sequence alignment of 984 positions. ML trees from single and combined COI and H3 markers all separate Brazilian and Mediterranean *Cratena* specimens into well-supported, reciprocally monophyletic clades (Figs 1, 2). In the ML consensus of both single COI (Fig. 1A) and concatenated (COI + H3; Fig. 2) trees, Brazilian *Cratena* and Mediterranean *C. peregrina* constitute well-supported sister clades (bootstrap support, BS = 99). This *Cratena* clade is sister to a clade with two *Sakuraeolis enosimensis* (BS = 100). Brazilian and Mediterranean *Cratena* also constitute separated and well-supported clades in the ML consensus tree of nuclear H3 (Fig. 1B), but for this gene Brazilian specimens form the sister clade to *S. enosimensis* (BS = 92), and together they are sister to the Mediterranean *C. peregrina* clade (BS = 99). The minimum uncorrected p-distance for COI between Mediterranean and Brazilian specimens was 17.19%, with a maximum of 0.67% among Brazilian specimens and 1.21% among Mediterranean specimens. ABGD analyses of the COI dataset, including *C. peregrina*, the Brazilian *Cratena* and *S. enosimensis* confirmed the two *Cratena* as distinct species when minimum prior intraspecific divergence (Pmin) was above 0.0045. For H3, there was no lower limit for Pmin, with the analysis also recognizing Mediterranean and Brazilian *Cratena* as distinct species. Birky's θ value for the Brazilian clade was 0.0163 and for the Mediterranean clade 0.0137; the K value was of 0.2 (Table 2). Being conservative and using the larger value of θ (see Birky, 2013), the K/θ value (i.e. 0.2/0.0163) is 12.26, clearly supporting the hypothesis of distinct species. COI haplotype network analyses in TCS resulted in independent parsimony networks for each of the three clades (*C. peregrina*, Brazilian *Cratena* and *S. enosimensis*). *Cratena peregrina* and Brazilian *Cratena* differed in 117 and 14 diagnostic characters of COI and H3, respectively. Molecular diagnosis is provided in the Supplementary material. Position numbers refer to the positions in the matrix deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S15602).

Morphology

Living specimens of *C. peregrina* can reach up to 50 mm in length and examined preserved specimens ranged from 13 to 17 mm. Living specimens of the Brazilian *Cratena* only reach up to 17 mm and preserved specimens from 2.5 to 6 mm. Specimens from the Mediterranean and Brazil show a whitish body with a
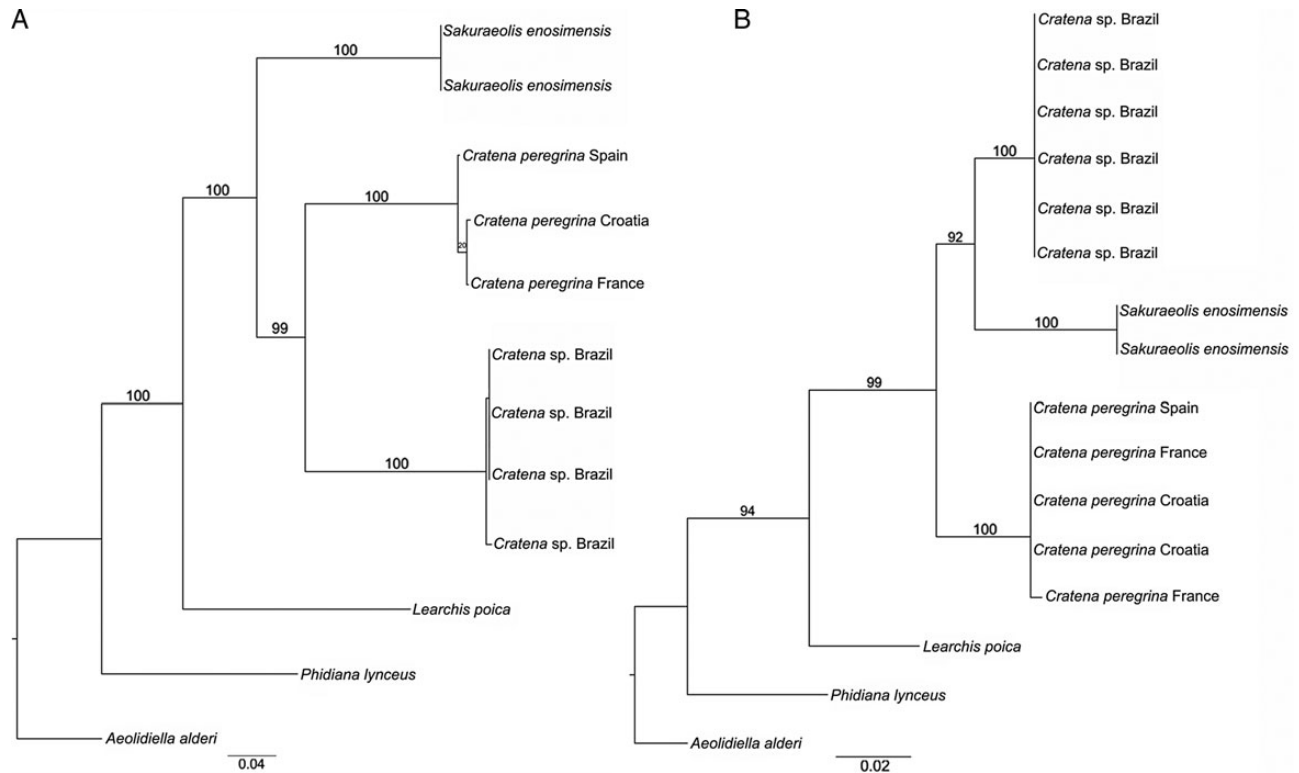
Figure 1. Maximum likelihood phylogenetic trees (1,000 replicates). Trees were rooted using *Aeolidiella alderi* as outgroup. **A.** Based on mitochondrial COI sequences. **B.** Based on nuclear H3 sequences. Bootstrap support values are shown above branches.

first row of cerata in an arc and subsequent cerata in rows. In Mediterranean and Brazilian specimens the cerata are translucent with orange to dark red digestive gland content, but in *C. peregrina* the apical region is usually bright blue (Fig. 3A). Specimens from both regions have rectangular orange spots between the rhinophores and the oral tentacles, but these are larger, quadrangular and laterally projecting on the head in Brazilian specimens (Fig. 3B). The rhinophores of *C. peregrina* have a translucent base, a large orange subapical band and a very small translucent white apical region. The rhinophores of Brazilian specimens are orange, with white distal region and tips (Fig. 3B). Both Mediterranean and Brazilian *Cratena* specimens have an oval jaw with denticulate border, but the jaw of Brazilian specimens has a depression in the dorso-central area. The denticles of the border are rounded and slightly pointed on Brazilian specimens (Fig. 4D). Mediterranean specimens have large triangular teeth, with prominent cusps in the border of the jaw (Fig. 4E, F). A 2.5-mm preserved Brazilian specimen (ZSM Mol 20110345) has a radula with 18 rachidian teeth, and a 3-mm specimen (ZSM Mol 20110338a) a radula with 17 rachidian teeth. A 10-mm preserved Croatian specimen (ZSM Mol 20100125) has a radula with 12 rachidian teeth, i.e. shorter in relation to body size. Radular teeth of Brazilian and Mediterranean specimens are similar, with a prominent central cusp and adjacent lateral cusps. Teeth of Brazilian specimens are triangular in shape and the lateral cusps are smaller near the central cusp and the margin of the teeth (Fig. 3A). The teeth of Mediterranean specimens are rounded and the lateral cusps are of similar length (Fig. 3B, C). The penis of Brazilian specimens is very large, protected by a penial sheath and with a basal glandular region (Fig. 4A). The penis of Mediterranean specimens is relatively small and lacks a basal glandular portion and surrounding sheath (Fig. 4B). The vas deferens of Brazilian specimens is cylindrical and subdivided into two main parts (Fig. 4A). In Mediterranean specimens it is pyriform, without

subdivision (Fig. 4B). The ampulla of Mediterranean specimens is more inflated than in Brazilian specimens.

SPECIES DELIMITATION

Our molecular phylogenetic study separates Brazilian and Mediterranean specimens into well-supported, reciprocally monophyletic clades. Results are congruent for a mitochondrial (COI) and an independently evolving nuclear marker (H3). Brazilian *Cratena* differ from the Mediterranean *C. peregrina* in 117 and 14 diagnostic characters of COI and H3, respectively (see Supplementary material), supporting the hypothesis of separately evolving lineages. To test whether these lineages show further subdivision or not, i.e. whether one or both might refer to species complexes, we used ABGD on the supposedly fast-evolving COI and on the nuclear H3. ABGD recovered *C. peregrina* and the Brazilian specimens as two different species in all analyses using standards values of the ABGD website, applying either Jukes–Cantor (JC69) or Kimura (K80)TS/TV models, but for COI the lower limit of Pmin was 0.0045, which is a very low value for intraspecific distances. This low value is related to the low intraspecific COI p-distances among specimens of *C. peregrina* (maximum 1.21%) and among specimens of the Brazilian *Cratena* (maximum 0.67%). Using ABGD for species delimitation requires data from sufficient specimens (>3–5) (Puillandre *et al.*, 2012), as herein. The resulting barcoding gaps in both rapidly evolving mitochondrial COI and slowly evolving nuclear H3 genes indicate more than just ephemeral reproductive isolation and are consistent with the unconnected COI haplotype networks. Different evolutionary lineages according to the unified species concept (De Queiroz, 2007) are interpreted as distinct species.

Supporting this hypothesis of long-lasting isolation, the minimum uncorrected COI p-distance of 17.19% between Brazilian and Mediterranean specimens is above the intraspecific divergences reported for molluscs in general (Hebert *et al.*, 2003)

and in studies focused on heterobranch sea slugs (e.g. Wilson, Schrödl & Halanych, 2009; Carmona *et al.*, 2011, 2013; Jörger *et al.*, 2012; but see Wägele *et al.*, 2010). We emphasize, however, that the establishment of a fixed threshold limiting intra- *vs* interspecific divergences should be avoided owing to the diverse evolutionary histories among heterobranchs, hindering the application of straightforward barcoding approaches (Jörger *et al.*, 2012; Jörger & Schrödl, 2013). Rather than relying mainly on genetic distance, we should focus on character-based approaches. Finding fixed mutations, i.e. diagnostic nucleotides in mitochondrial and nuclear genes as herein, can provide strong evidence for separate species (e.g. Ornelas-Gatdula *et al.*, 2012; Jörger & Schrödl, 2013).

The recently established K/θ ratio method measures the sequence difference between putative species (well-supported clades on single gene trees) and compares it with differences within species, applying population-genetic theory concepts (Birky, 2013). It is thus gene and tree dependent, but avoids relying on intuition to decide when branches of a tree and support values are enough to separate species (Birky, 2013). According to coalescent theory, K/θ ratios >4 in mitochondrial genes distinguish at 95% probability level sister clades composed

of 5 *vs* single specimens, and K/θ ratios >4.2 can delimit a singleton from a sister doubleton (Birky, 2013). Such tolerance to undersampling, if confirmed by empirical studies, would be in contrast to other model-based methods such as GMYC, which to produce reliable results need the inclusion of a large number of samples (see Hamilton *et al.*, 2014). This condition is seldom fulfilled when working with rare or elusive animals (see Jörger *et al.*, 2012). The K/θ ratio of 12.26 obtained herein (Table 2) is far above the limit $(K/\theta < 4)$ for conspecificity (Birky, 2013) and thus provides evidence of two distinct *Cratena* species.

Discussing advantages and limitations of his method, Birky (2013) recommends usage of single (mitochondrial) genes because of their fast evolution. However, different gene trees may show incongruences, as is the case presented here (Figs 1, 2). We emphasize that gene trees alone, even if reconstructed correctly, do not necessarily correspond to species trees. Another essential problem for species delimitation studies refers to usually inadequate coverage of genetic diversity of populations (Bergsten *et al.*, 2012) across the entire, usually unknown, geographic range of the species. An appropriate method to detect statistically even recently diverged, unsorted species from limited specimen samples is Bayesian species delineation (BPP) (Yang & Ranala, 2010; Zhang *et al.*, 2011), but this needs multiple, independently evolving sequence markers (see Jörger *et al.*, 2012). Considering a trade-off between efforts and costs on the one hand, and resolution and reliability of molecular results on the other, initial analyses of few loci (both mitochondrial and nuclear) with multiple appropriate methods should perform well in unambiguous cases, such as that of the *Cratena* species presented herein.

Morphology also offers a potentially fast-evolving suit of more or less independently evolving characters, which are relatively easy collected from a wide range of samples, including photographs of specimens from remote places and museum specimens not suitable for genetic study. The individual and combined significance of characters is, however, difficult to assess quantitatively. We show that there are several slight but consistent morphological differences between Brazilian and Mediterranean *Cratena* specimens, in body sizes, coloration and internal morphology. We consider such congruent, apparently fixed differences as proxies suggestive of reproductive isolation. Clear differences observed in the reproductive system point to intrinsic reproductive barriers. The studied *Cratena* specimens belong to allopatric coastal populations, separated by the Atlantic Ocean, without any know populations in between. In the absence of fossils or well-established molecular clocks for nudibranchs, geographical distance and assumption of some hydrographic continuity could also be suggestive of permanent, ancient (rather than recently established) reproductive isolation.

In summary, there are several lines of evidence for considering Brazilian *Cratena* specimens specifically distinct from *C. peregrina*, i.e. forming separately evolving lineages as required under the commonly used unified species concept (De Queiroz, 2007). However, limited data are available on the geographical distribution ranges of most nudibranch species, and intermediate *Cratena* populations between Brazil and the Mediterranean may exist but have not yet been discovered. Furthermore, nudibranch larvae are usually pelagic, with considerable dispersal ability, and there are some other sea slug species with a molecularly confirmed amphiatlantic distribution (Carmona *et al.*,
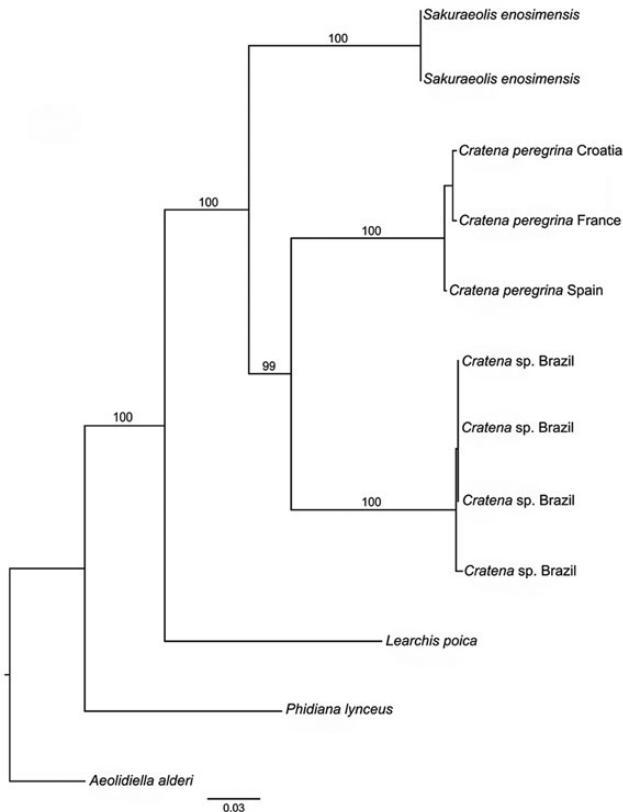
Figure 2. Maximum likelihood phylogenetic tree (1,000 replicates) rooted using *Aeolidiella alderi* as outgroup. Based on cytochrome *c* oxidase subunit I and H3 concatenated sequences. Bootstrap support values are shown above branches.

Table 2. K/θ ratio dependent parameters (see Birky, 2013 for detailed information).

Clade	Number of sequences (*n*)	Pairwise difference (*d*)	Nucleotide diversity (π)	θ	K2P[a]	K/θ
Brazilian *Cratena* clade	4	0.004	0.005332	0.0163	0.2	12.269
Cratena peregrina clade	3	0.009	0.0135	0.0137	0.2	14.598

[a]Corrected values of Kimura 2-parameter distances.

Figure 3. Living specimens. **A.** *Cratena peregrina*, Naples, Italy, showing the most common colour pattern of the species. **B.** *Cratena minor* n. sp., holotype (CMPHRM 4026B), Ceará, Brazil.

2013; Cámara *et al.*, 2014). As always with allopatric populations in nonexhaustively studied taxa, interpretation as distinct species or not depends on the amount and significance of detected differences tolerated by the taxonomist, and no consensus on best practice has yet been reached.

General guidelines for integrative species delimitation were recently proposed. Padial *et al.* (2010) discussed different schemes according to the accumulation of evidence. In allopatry, two groups of specimens with a difference in a taxonomic character, such as colour pattern or size, should be representatives of different species if they present congruent differences in a character mediating sexual isolation (Padial *et al.*, 2010: fig. 3D). This apparently occurs in the Mediterranean and Brazilian specimens of *Cratena* studied here, which show remarkable differences in their reproductive systems (Fig. 4). Investigating reptiles, Miralles *et al.* (2011) cited three lines of evidence: (1) mtDNA: presence of independent parsimony networks with a connection limit of 95%; (2) nDNA: absence of shared haplotypes and (3) morphology: detection of at least one fixed diagnostic character state. Miralles *et al.* (2011) pragmatically required two of these three lines of evidence to be fulfilled, to indicate the occurrence

of two distinct species. In our case, these three lines are all fulfilled (independent parsimony networks for COI; absence of shared haplotypes in H3 and fixed morphological differences).

As conducted on problematic acochlidian heterobranchs by Jörger *et al.* (2012), we recommend the investigation of several individuals covering populations from different regions, the application of a variety of appropriate analytical tools (see above) and the combination and integration of evidence from different datasets. These should include mitochondrial and nuclear genes, in addition to anatomy, the last with special emphasis on reproductive features.

As a result of evidence from our molecular study, including the phylogenetic hypothesis with well-supported, reciprocally monophyletic clades in the two independent markers (COI and H3), the presence of fixed diagnostic characters, the ABGD analysis and the K/θ ratio, we conclude that the Brazilian specimens do not belong to the Mediterranean *Cratena peregrina*. The molecular study confirms that the morphological and body colour differences are not an expression of intraspecific variation within *C. peregrina*. Therefore, we conclude that *C. peregrina* is not present in Brazil; instead the Brazilian specimens belong to a new species which is described below.
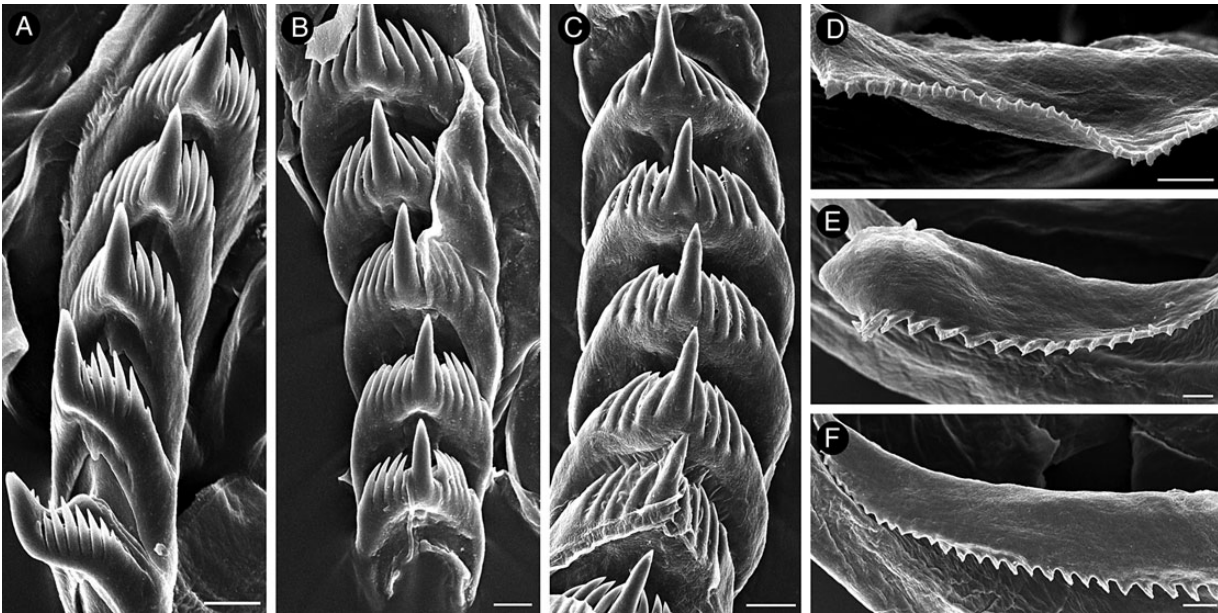
Figure 4. SEM micrographs. **A.** Rachidian teeth of *Cratena minor* n. sp. (ZSM Mol 20110345). **B, C.** Rachidian teeth of *C. peregrina* from Croatia (ZSM Mol 20100125) and France (ZSM Mol 20020957), respectively. **D.** Border of jaw of *C. minor* n. sp. (ZSM Mol 20110345). **E, F.** Border of jaw of *C. peregrina* from Croatia (ZSM Mol 20100125) and France (ZSM Mol 20020957), respectively. Scale bars: **A, B, D, E** = 10 μm; **C, F** = 20 μm.
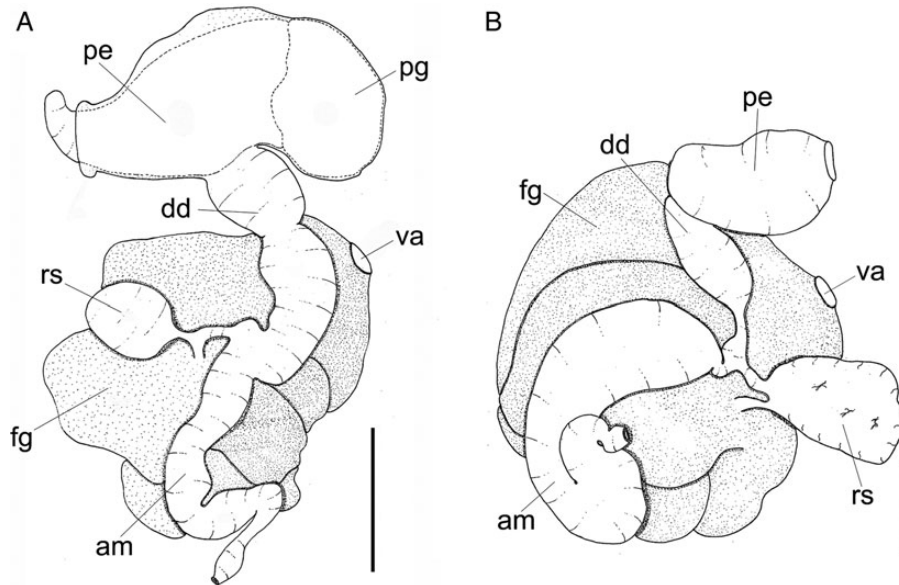

Figure 5. Reproductive system. **A.** *Cratena minor* n. sp. (CMPHRM 3728B). **B.** *C. peregrina* (ZSM Mol 20130772). Abbreviations: am, ampulla; dd, deferent duct; fg, female gland; pe, penis; pg, penial gland; rs, receptaculum seminis; va, vagina. Scale bars = 0.5 mm.

SYSTEMATIC DESCRIPTION

Facelinidae Bergh, 1889
***Cratena* Bergh, 1864**

***Cratena minor* new species**
(Figs 3B, 4A, D, 5A)

Cratena cf. *peregrina*—Galvão Filho, Meirelles & Mathews-Cascon, 2011: 105.
? *Cratena* cf. *peregrina*—Valdés *et al*., 2006: 258.

Types: Holotype (CMPHRM 4026B, intact): Praia da Caponga, Cascavel, Ceará, Brazil, intertidal, on hydroid *Eudendrium carneum*, 17 mm long alive, 12 March 2009, leg. H. C. Galvão Filho. Paratypes: (CMPHRM 4027B, 1 spec., intact) Praia da Caponga, Cascavel, Ceará, Brazil, intertidal, on hydroid *E. carneum*, 15 mm long alive, 12 March 2009, leg. H. C. Galvão Filho; (ZSM Mol 20110345, 1 spec., dissected), Ponta Itapessoca, Pernambuco, Brazil, 2.5 mm long preserved, 15 March 2011, leg. M. Schrödl., GenBank acc. no. KJ940476 and KM079346 (MZSP 116702, 1 spec.), Ponta Itapessoca, Pernambuco, Brazil, 3 mm long preserved, 3–10 m., 03 March 2011, leg. R. Carvalho and M. Schrödl., GenBank acc. no. KJ940479 and KM079345.

Additional material: (CMPHRM 3728B, 2 specs, dissected) Praia da Caponga, Cascavel, Ceará, Brazil, intertidal, on the hydroid *E. carneum*, 5 and 4.5 mm long preserved, 12 January 2009, leg. H. C. Galvão Filho. (CMPHRM 3729B, 1 spec., dissected) Praia da Caponga, Cascavel, Ceará, Brazil, intertidal, on the hydroid *E. carneum*, 5 mm long preserved, 13 September 2011, leg. H. C. Galvão Filho.

ZooBank registration: urn:lsid:zoobank.org:act:3301936E-7613-4EFD-80AD-75AD7DB555E6.

Etymology. From the Latin *minor*, smaller, due to the small size of the species in comparison with the similar Mediterranean *C. peregrina*.

Molecular diagnosis: Cratena minor n. sp. differs from *C. peregrina* in 117 and 14 diagnostic characters of COI and H3, respectively (see Supplementary material).

Diagnosis: Small aeolid, up to 17 mm long; oral tentacles long, 1/3 body length; rhinophores smooth; precardiac cerata in arches, postcardiac cerata in rows; gonopore below first group of cerata; anus anterior to second group of cerata. Radula (Fig. 4A): 18 rachidian teeth (ZSMMol 20110345, 2.5 mm preserved specimen); teeth triangular, prominent central cusp smooth; up to eight small lateral cusps, lateral cusps smaller near central cusp and at border of teeth. Jaw plate (Fig. 4D): ovate, with slight dorsal indentation, cutting edge projecting in short triangular area, denticulate border with single row of bluntly pointed denticles. Seminal receptacle small, rounded on short stalk; penis large, with basal glandular portion (Fig. 5A). Body white; oral tentacles, head and foot translucent white; pair of almost quadrangular orange spots laterally on head, between rhinophores and oral tentacles; rhinophores with translucent base, a median orange band and white distal portion; cerata translucent with red to dark red digestive gland content; cnidosac white (Fig. 3B).

Distribution. Ceará and Pernambuco, northeastern Brazil (Galvão Filho *et al.*, 2001; present study). Possibly also Florida (Valdés *et al.*, 2006).

Remarks. Brazilian specimens are allocated to the genus *Cratena* due to the disposition of cerata (first group in arc, subsequent groups in rows), the radular tooth shape and due the absence of a stalked penial gland, which is present in *Sakuraeolis* (Baba & Hamatani, 1965; Rudman, 1980). However, Brazilian specimens clustered with *S. enosimensis* in our nuclear gene H3 phylogenetic analysis. The delimitations of genera within the Facelinidae have been a matter of debate for a long time (see Edmunds, 1970; Miller, 1974; Edmunds & Just, 1983) and require a comprehensive review based on a molecular approach. Apart from the most similar species *C. peregrina*, some other species resemble *C. minor*. *Cratena scintilla* Ortea & Moro, 1998 from the Cape Verde Islands is very similar to *C. peregrina*, differing in the presence of an orange line on the side of the body, white marks on the tips of the cerata and orange base of the oral tentacles (Ortea & Moro, 1998). Another similar species is *C. kaoruae* Marcus, 1957, originally described from São Paulo, southeastern Brazil. Marcus (1957) described the first three group of cerata in arches ('horseshoe-shaped') and the subsequent ones in oblique rows, while only the first group of cerata is arranged in an arch in *C. minor*. Marcus (1957) mentioned the presence of orange pigment on the sides of the head, although not in conspicuous spots as occur in *C. minor* and *C. peregrina*. Marcus (1972) synonymized *C. kaoruae* with *C. pilata* (Gould, 1870) from Massachusetts, based mostly on similarities in the morphology of the reproductive system. Ortea *et al.* (2005) rejected this synonymy and reallocated *C. kaoruae* to the

genus *Facelina*, due to the morphology of the radular teeth and the arrangement of cerata. Ortea *et al.* (2005) provided a photo of a specimen of *F. kaoruae* from Cuba, which clearly differs from *C. minor*. Another western Atlantic species, *C. piutaensis* Ortea, Caballer & Espinosa, 2003, differs in general body colour pattern and external morphology, and has recently been placed in the genus *Anetarca* by Ortea *et al.* (2005).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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3. RESULTS

<u>Chapter 8</u>

Pola M, **Padula V**, Gosliner TM, Cervera JL (2014) Going further on an intricate and challenging group of nudibranchs - description of five new species and a more complete molecular phylogeny of the subfamily Nembrothinae (Polyceridae). *Cladistics* **30**: 607-634.

Going further on an intricate and challenging group of nudibranchs: description of five novel species and a more complete molecular phylogeny of the subfamily Nembrothinae (Polyceridae)

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Abstract

Nembrothinae is a colourful subfamily of nudibranch polycerids, which despite its large size and striking appearance, needs to be more thoroughly studied. The available scientific information about this subfamily is very recent, and pictures of living undescribed species become available every day. Nevertheless, the lack of associated material for morphological, anatomical, and molecular analysis results in scarce additional studies. In this paper, five novel species are described: *Roboastra ernsti* sp. nov., *Roboastra nikolasi* sp. nov., *Tambja brasiliensis* sp. nov., *Tambja crioula* sp. nov., and *Tambja kava* sp. nov. In addition, *Tambja divae* (Marcus, 1958), a species previously known only from the original description, is redescribed and additional data and comments on *Tambja* cf. *amakusana* Baba, 1987 and *Tambja marbellensis* Schick and Cervera, 1998 are provided. Molecular data (H3, *COI* and *16S* genes) for all these novel species and some additional ones were obtained and included in a previous molecular database. Maximum-likelihood, maximum-parsimony and Bayesian analyses were carried out. The phylogeny presented here has revealed Nembrothinae to be an intricate and challenging group of nudibranchs to study. Intermediate missing species seem to be critical to understanding the evolutionary relationships within this group.
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Introduction

Traditionally, the subfamily Nembrothinae Burn, 1967 has comprised the genera *Nembrotha* Bergh, 1877, *Roboastra* Bergh, 1877, and *Tambja* Burn, 1962. Most species were described in the 19th and 20th centuries, based on the study of one or two specimens, many with incomplete or misleading descriptions. The lack of detailed information and the existence of several undescribed species led to the undertaking of a comprehensive study on the taxonomy and the phylogeny of the subfamily Nembrothinae (Pola et al., 2003, 2005a,b,c, 2006a,b,c, 2007, 2008a,b). As result of these

studies, 12 species were described, three of *Roboastra*, seven of *Tambja*, and two of *Nembrotha*. In addition, some specific names were synonymized and *Roboastra arika* Burn, 1967 and *Nembrotha caerulea* Eliot, 1904 were considered as *nomina dubia*. One of the most relevant contributions of these studies concerns the phylogenetic relationships of the subfamily, its genera, and species (Pola et al., 2007). Thus, the monophyly of the Nembrothinae is supported but only the genus *Nembrotha* was recovered as monophyletic. The phylogenetic position of *Tambja tentaculata* Pola et al., 2005a,b,c prevents the recovery of the monophyly of the genus *Roboastra* in all analyses. This situation led Pola et al. (2008b) to assume one of their own taxonomic alternatives suggested a year previously (Pola et al., 2007), allocating *T. tentaculata* to *Roboastra*. Thus,

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grooved and well-developed oral tentacles are the single synapomorphy recognized for *Roboastra*. In contrast, the genus *Tambja* was not recovered as monophyletic in all analyses, as *Roboastra* is nested within *Tambja*.

Although anatomical information for most Nembrothinae has been updated over the last 10 years, Pola et al. (2007, 2008b) could not check additional material and did not have access to well-preserved specimens of some species for anatomical and molecular studies. The results and hypothesis of Pola et al. (2007, 2008b) will remain untested until more specimens and species are included. Field trips to different regions of the world have provided material of some of these species and also material of undescribed nembrothid species. In this paper we redescribe *Tambja divae* (Marcus, 1958), a species previously known only from the original description, provide additional data and comments on *Tambja marbellensis* Schick and Cervera, 1998, and describe five new nembrothid species. Moreover, molecular data from an additional 11 species, including the new species, were obtained and included in the new phylogenetic study presented herein. All these new data allowed a reassessment of the phylogenetic relationships of Nembrothinae, provided evidence of unexpected intraspecific variation of some traditional taxonomic characters, and indicate that systematics of Nembrothinae are even more complex than previously thought.

Material and methods

The specimens described in this study were collected in several field trips to Vanuatu, Brazil, Malaysia, Mexico, and Cape Verde Archipelago and are currently located at the California Academy of Sciences Department of Invertebrate Zoology and Geology in San Francisco (CASIZ), the Museo Nacional de Ciencias Naturales in Madrid (MNCN), the Colección Nacional de Moluscos de Instituto de Biología de la Universidad Autónoma de México (CNMO), the Museu de Zoologia da Universidade de São Paulo (MZSP) and the Museu Nacional/Universidade Federal do Rio de Janeiro (MNRJ), Brazil.

Specimens were collected by scuba diving. Features of living animals were recorded in the field and from photographs. The specimens were preserved directly in 75–100% EtOH except for some specimens that were preserved in formalin. From the latter specimens small tissue pieces from the foot were directly preserved in 95% EtOH for molecular studies. To undertake the morphological descriptions the specimens were dissected under a microscope and external and internal features were drawn with the help of a camera lucida. The buccal mass was removed and soaked in a 10% sodium hydroxide solution to dissolve the connective and

muscle tissue, leaving only the radula and the labial cuticle. The penis was critical point dried. The coated radula, labial cuticle and penis of each specimen were examined and images were obtained using scanning electron microscopes (Leo 1450 VP, Fei Quanta 200, and Hitachi S-3000N).

Genomic DNA was extracted from small pieces of foot tissue for most samples using Qiagen DNeasy Tissue Kits (Qiagen, Valencia, CA, USA). Amplification of DNA was conducted on a BioRad MyCycler[T] Thermocycler (software version 1.065). Partial sequences of the mitochondrial genes *cytochrome c oxidase subunit I* (658 bp) and *16S rRNA* (485 bp) and the nuclear gene *Histone 3* (328 bp) were amplified using primer pairs LCO1490 and HCO2198 (Folmer et al., 1994), 16Sar-L and 16Sbr-H (Palumbi et al., 1991), and H3a F and H3a R (Colgan et al., 1998), respectively. PCR amplifications were carried out in a 25-µL reaction volume including 1 µL of 10 × PCR buffer, 0.2 µL dNTPs (10 mM stock), 1.5 µL MgCl (25 mM stock), 0.025 µL Taq (1.25 units/µL)-Apex, 0.2 µL of each primer (25 µM stock), and 1 µL of genomic DNA. Standard PCRs for COI consisted of: an initial denaturing step at 94 °C for 3 min; 40 cycles of denaturing at 94 °C for 30 s and annealing at 48–50 °C for 30 s; and final extension at 72 °C for 5 min. The partial 16S amplifications followed the following parameters: an initial denaturing step at 94 °C for 3 min; 39 cycles of denaturing at 94 °C for 30 s and annealing at 50–52 °C for 30 s; and extension at 72 °C for 2 min and 25 °C for 2 min. Finally, the PCR conditions for the H3 amplification consisted of an initial denaturing step at 94 °C for 3 min; 35 amplification cycles (94 °C for 35 s, 50 °C for 1 min, and 72 °C for 75 s), and a final step at 72 °C for 2 min. Double-stranded amplified product was electrophoresed in a 0.5% TBE agarose gel stained with ethidium bromide. Amplified products were purified with ExoSAP-IT (usb.affymetrix.com). Ccle-sequencing reactions were performed using ABI Prism Big Dye Terminator (Applied Biosystems Foster City, CA, USA) (total volume 10 µL) and analysed using the automated sequencers ABI 3130 and 3730XL (Applied Biosystems). All new DNA sequences have been deposited in GenBank (Table 1).

COI, 16S and *H3* gene sequences were added to a previous molecular dataset (Pola et al., 2008b). DNA sequences were assembled and edited using Geneious Pro 4.7.6. (Drummond et al., 2009). All the sequences were checked for contamination with BLAST (Altschul et al., 1990) implemented in the GenBank database. Geneious and MAFFT (Katoh et al., 2009) were employed to align the sequences, using the default settings in both programs. The alignments were checked by eye using MacClade version 4.06 (Maddison and Maddison, 2005). Protein-coding sequences were translated into amino acids for confirmation of alignment.

Table 1
Additional specimens used in this study for molecular purposes, collection sites, voucher, and GenBank accession numbers

Species	Locality	Voucher	GenBank accession numbers		
			COI	16S	H3
Roboastra ernsti	Brazil, São Paulo, Ilha da Serrania	MZSP 103252	KJ999211	KJ999190	KJ999231
Roboastra ernsti	Brazil, Rio de Janeiro, Cabo Frio	MNCN 15.05/60095	KJ999212	KJ999191	KJ999232
Roboastra nikolasi	Vanuatu, Espiritu Santo Island	CASIZ 177031	KJ999213	KJ999192	KJ999233
Roboastra nikolasi	Malaysia, South China Sea, Jubilee Shoal	CASIZ 176778	KJ999214	KJ999193	KJ999234
Tambja brasiliensis	Brazil, São Paulo, Ilha de São Sebastião	CASIZ 180372	KJ999215	KJ999194	KJ999235
Tambja brasiliensis	Brazil, São Paulo, Ilha de São Sebastião	CASIZ 180373	KJ999216	KJ999195	KJ999236
Tambja brasiliensis	Brazil, São Paulo, Ilha da Serrania	MZSP 103242	KJ999217	KJ999196	KJ999237
Tambja brasiliensis	Brazil, São Paulo, Ilha da Serrania	MZSP 103243	KJ999218	KJ999197	KJ999238
Tambja brasiliensis	Brazil, São Paulo, Ilha da Serrania	MZSP 103244	KJ999219	KJ999198	KJ999239
Tambja brasiliensis	Brazil, São Paulo, Ilha da Serrania	MZSP 103245	KJ999220	KJ999199	KJ999240
Tambja brasiliensis	Brazil, São Paulo, Ilha da Serrania	MZSP 103246	KJ999221	KJ999200	KJ999241
Tambja crioula	Cape Verde, Ilha de Santiago, Tarrafal	CASIZ 180377	KJ999222	KJ999201	KJ999242
Tambja divae	Brazil, São Paulo, Ilha de Buzios	MZSP 103230	KJ999223	KJ999202	KJ999243
Tambja kava	Vanuatu, Western Aoré Island	CASIZ 178792	KJ999224	KJ999203	KJ999244
Tambja marbellensis	Portugal, Setubal, Outão	CASIZ 180379	HM162689	KJ999204	HM162505
Tambja olivaria	Vanuatu, Espiritu Santo Island	CASIZ 178679	KJ999225	KJ999205	KJ999245
Tambja stegosauriformis	Brazil, Rio de Janeiro, Ilha do Papagaio	CASIZ 180370	KJ999226	KJ999206	KJ999246
Tambja cf. *tenuilineata*	Mexico, Yucatan Peninsula, Progreso Port	CNMO 4387	KJ999227	KJ999207	X
Tambja victoriae	Vanuatu, Espiritu Santo Island	CASIZ 176853	KJ999228	KJ999208	KJ999247
Tambja zulu	South Africa, Kwazulu-Natal	CASIZ 181518	KJ999229	KJ999209	KJ999248
Tambja zulu	South Africa, Kwazulu-Natal	CASIZ 181519	KJ999230	KJ999210	KJ999249

Saturation was visually inspected in MEGA 5.0 (Tamura et al., 2011) by plotting for all specimens including the outgroup the total number of pairwise differences (transitions and transversions) against uncorrected p-distances. For the COI and H3 genes, saturation was further examined separately for the first, second, and third codon positions.

The most variable regions from the 16S rRNA alignment were removed using both the default settings and the standard options for stringent and less stringent selection in Gblocks (Talavera and Castresana, 2007). Excluding "indel-rich" regions, the tree was in general poorly resolved with lower node support. Therefore, final analyses were performed with all bases included. Sequences of *COI*, *16S*, and *H3* were trimmed to 658, 415, and 328 bp, respectively.

Individual gene analyses (*COI*, *16S*, and *H3*) and two different concatenated analyses (*COI+16S* and *H3+COI+16S*) were performed. The *COI+16S* dataset included four molecular partitions (COI-1st, COI-2nd, COI-3rd, 16S). The *H3+COI+16S* dataset included seven partitions (H3-1st, H3-2nd, H3-3rd, COI-1st, COI-2nd, COI-3rd, 16S). To test for conflicting phylogenetic signal between genes, the incongruence length difference (ILD) test (Farris et al., 1994) was conducted as the partition homogeneity test in PAUP* 4.0b10 (Swofford, 2002). Test settings consisted of 10 random stepwise additions (100 replicates) with TBR branch swapping.

Sequence analysis was based on the maximum-parsimony (MP) and maximum-likelihood (ML) optimality criteria. The best-fit models of evolution for each gene were determined using the Akaike information criterion (Akaike, 1974) implemented in MrModeltest 2.3 (Nylander, 2004). The selected models by positions were as follows: GTR+I+G for COI-1st and COI-3rd, F81 for COI-2nd, GTR for H3-1st and H3-3rd, GTR+G for H3-2nd, and HKY+I+G for 16S. The MP analyses were performed by heuristic searches under TBR branch swapping and 100 random replicates using PAUP* 4.0b10 (Swofford, 2002). All characters were left unweighted and gaps were treated as missing characters (Ogden and Rosenberg, 2007). We used non-parametric bootstrapping (1000 pseudoreplicates) in the MP analyses to assess nodal support (Felsenstein and Kishino, 1993). ML analyses were performed using the software RAxML v7.0.4 (Stamatakis, 2006) and node support was assessed with non-parametric bootstrapping with 50 000 replicates, random starting trees, and parameters estimated from each dataset under the model selected for the original dataset. Bayesian inference (BI) analyses were also conducted using MrBayes version 3.1.2b (Ronquist and Huelsenbeck, 2003) for ten million generations and four chains. Markov chains were sampled every 1000 generations. Of the resulting trees, 2500 were discarded as "burn in". The models implemented were those estimated with MrModeltest 2.3. The combined dataset was partitioned among genes and the "unlink" command was used to allow all parameters to vary independently within each partition. Only nodes supported by bootstraps values ≥ 70 (Hillis and Bull, 1993) and posterior probabilities ≥ 0.95 were considered statistically significant (Alfaro et al., 2003). *Crimora lutea* Baba, 1949 was chosen to root the tree as well as in Pola et al. (2008b).

To compare the genetic distances amongst specimens of Nembrothinae, we calculated the pairwise uncorrected p-distances for *COI* using PAUP* 4.0 b 10.0. All codon positions were considered for the analysis.

Nomenclatural acts

This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub:70DC7FF1-3C00-4DB3-8ED1-0F3F87462EA1.

Systematics
Family Polyceridae Alder and Hancock, 1845
Subfamily Nembrothinae Burn, 1967

Genus *Roboastra* Bergh, 1877

Type species. Nembrotha gracilis Bergh, 1877: 458, pl. 56, Figs 11–17.

***Roboastra ernsti* sp. nov. (Figs 1a–d, Fig. 2, Fig. 3a)**

LSID. urn:lsid:zoobank.org:act:B39DDF71-BE87-47B2-A712-C2954F093594.

Type material. Holotype: MZSP 103252, Brazil, São Paulo, Ilha da Serrania (23°48′68″S, 45°13′72″W), 19 January 2012, 1 specimen, 80 mm alive, 8 m depth, collected by V. Padula. *Paratype*: MNCN 15.05/60095, Brazil, Rio de Janeiro, Cabo Frio, Ilha dos Pargos, Enseada da Meia Lua (22°51′25.25″S, 41°54′34.20″W), 23 April 2010, 1 specimen, 60 mm alive, 8 m depth, collected by V. Padula.

Additional material. MZSP 37978, Brazil, Rio de Janeiro, Macaé, 6 March 1986, 1 specimen, 35 mm (preserved), col. Eurico de Oliveira leg., Id. Marcus.

Etymology. This species was one of the last nudibranchs dissected by Eveline Marcus and labelled as "*Roboastra ernsti*" (in honour of her late husband, Ernst Marcus) but she did not have enough time to describe it before her death in 1990. We decided to use the name "*Roboastra ernsti*" in honour to both Eveline and Ernst Marcus.

Distribution. Thus far this species is known from south-eastern and southern Brazil, from Rio de Janeiro to Santa Catarina states (Krause, 2003; Debelius and Kuiter, 2007; present study).

External morphology. (Fig. 1a–d). Body elongate and limaciform with a long and pointed posterior end of the foot. The animals alive reach 80 mm in length. The body surface is strongly wrinkled but smooth. The foot is linear. The head is rounded with a pair of conical, completely retractile, perfoliate rhinophores with approximately 35–40 tightly packed lamellae. The oral tentacles are strongly developed and dorsolaterally grooved along part of their length. There are five non-retractile tripinnate gill leaves; the three anteriormost gill leaves are more highly developed. The gill forms a semicircle surrounding the anal papilla, which is elevated. The genital pore opens on the right side, midway between the gill and the rhinophores. The ground colour is dark green; some areas such as the anterior portion of the body, above the eyes, and the border of the foot can be dark blue. A series of irregular longitudinal yellow lines cover the entire body, except for a short area in the transition between the dorsum and body laterals. The yellow lines are most numerous on the lateral surface of the body, with up to 14 lines, most of which are incomplete. A yellow line may border internally the dorsal region and in some specimens also the base of rhinophore sheaths. One to three lines run from the rhinophores to the gill, ramifying in the gill branches. Oral tentacles are dark green proximally, near to the mouth, and dark blue distally. Rhinophores have a dark blue column, lamellae varying from dark blue to dark green, covered with yellowish pigmentation in some specimens. Rhinophore tips are blue. Base of the gill branches with the same colour of the body; distally they are dark blue/green with light leaves.

Internal morphology. The three available specimens were dissected, but the Marcus' specimen was already missing the buccal bulb. However, Ev. Marcus notes were found and her description and drawings of the teeth are now available (see Supplementary material). The anterior digestive tract begins with a short, thick-walled muscular oral tube, which continues into the buccal mass, and is relatively small compared with the size of the animal. At their junction, a pair of slender elongate pouches opens into the digestive system. The salivary glands are short and thick, opening on to the buccal mass and flanking the oesophagus. The labial cuticle is weak, without any armature. There is a well-developed blood gland, which is granular in texture. The radular formula is $34 \times 4.1.1.1.4$ for both holotype and paratype specimens (Fig. 2a) and $100 \times 4.1.1.1.4$ for the Marcus specimen. The rachidian tooth is broad, thin and very arched at its base, with three well-differentiated cusps almost similar in size, the central cusp even longer than the laterals (Fig. 2a,b). The inner
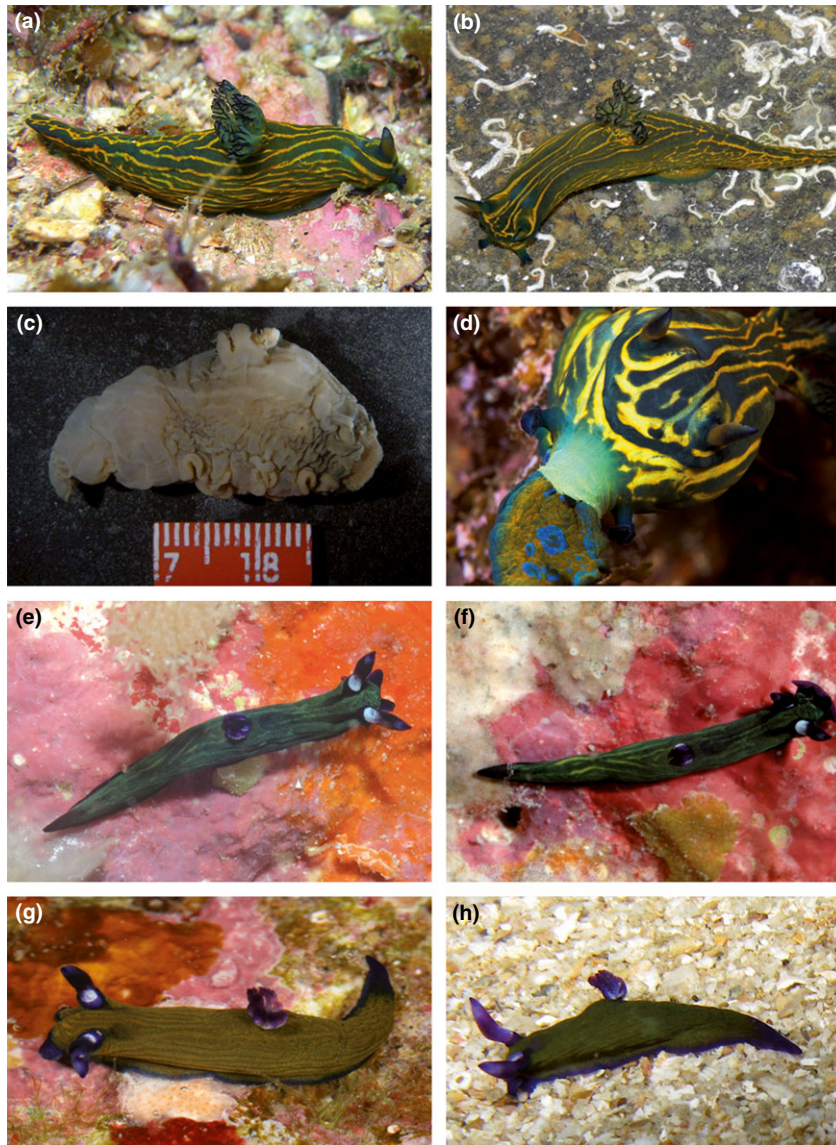
Fig. 1. Photographs of the living animals. (a–d) *Roboastra ernsti* sp. nov., Brazil. (a) MNCN 15.05/60095 (Paratype), 60 mm in length, Rio de Janeiro (photo: Vinicius Padula). (b) MZSP 103252 (Holotype), 80 mm in length, São Paulo (photo: Vinicius Padula). (c) MZSP 37978, Rio de Janeiro (photo: Carlo Cunha). (d) *Roboastra ernsti* sp. nov. feeding on *Tambja stegosauriformis* (photo: Ulisses Turati). (e–h) *Roboastra nikolasi* sp. nov. (e, f) CASIZ 177031, 15 mm in length, Vanuatu (photo: Yolanda Camacho). (g) CASIZ 176778, 15 mm in length, Malaysia (photo: T. M. Gosliner). (H) Singapour (photo: Sonneblume).

lateral tooth is large and very curved, having two very well-developed cusps. The inner cusp is simple and larger than the outer one, which is very slender. From a different angle, it is possible to observe that the upper cusp has a large protrusion in the anterior upper area while at the same area but in the lower part of the cusp there is a small projection (Fig. 2c,d). The remaining lateral radular teeth are more or less quadrangular, lack cusps or denticulation, and become smaller near the margin (Fig. 2a–c).

The reproductive system is triaulic (Fig. 3a). The hermaphroditic duct widens into a large S-shaped ampulla, which has thick walls. The ampulla narrows into the postampullary duct, which bifurcates into the vas deferens and oviduct. The short oviduct enters the female gland mass. The deferent duct, which lacks a morphologically well-differentiated prostate, is long and coiled, ending in a dilated and darkly pigmented penial atrium. The duct has a uniform width, but is slightly narrower and thinner in the prostatic part. The penis is located within the distal end of this muscular portion and is armed with at least three different kinds of hooked and chitinous spines arranged in helicoidal rows (Fig. 2e). The types of spines and their
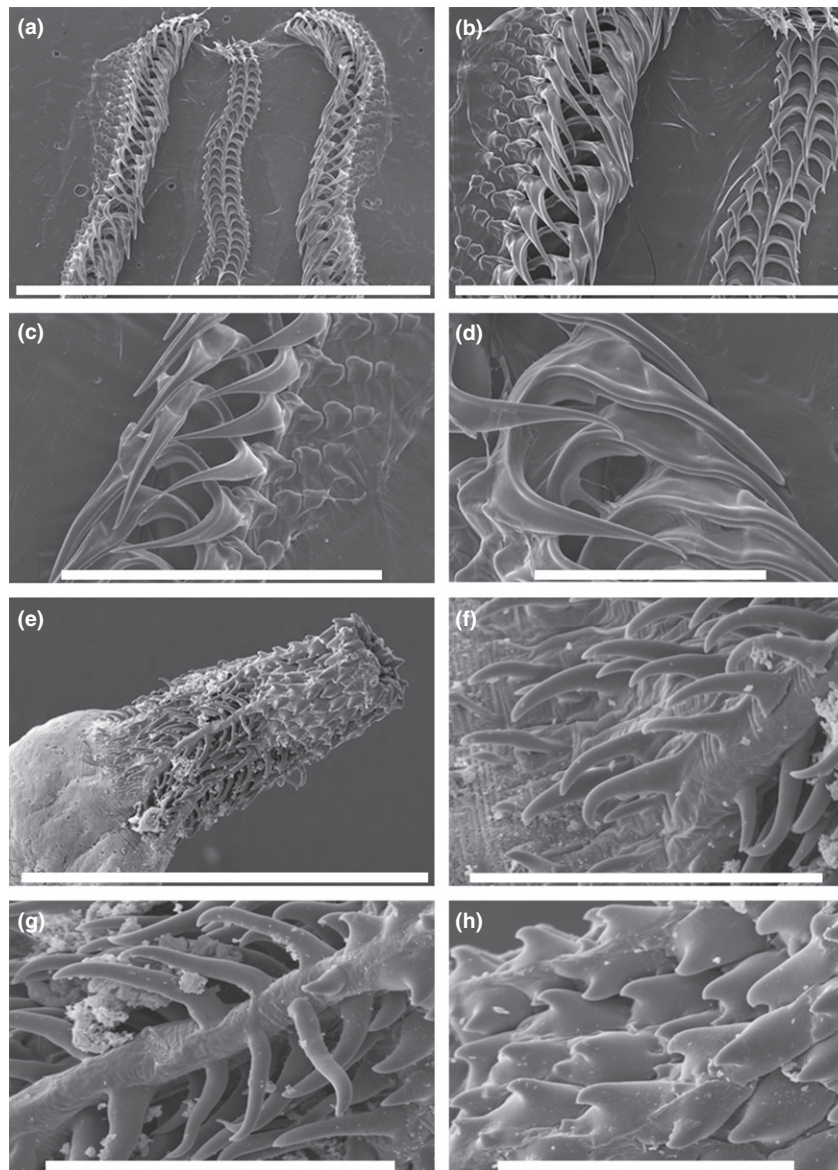
Fig. 2. Scanning electron micrographs of *Roboastra ernsti* sp. nov. (a–d) MZSP 103252 (Holotype). (a) Radula. Scale bar: 5 mm. (b) Left half of the radula. Scale bar: 2 mm. (c) Lateral teeth. Scale bar: 1 mm. (d) Innermost lateral teeth. Scale bar: 500 μm. (e–h) MNCN 15.05/60095 (Paratype). (e) Penis. Scale bar: 300 μm. (f–h) Details of the three different kinds of spines. Scale bars: 50 μm.

arrangement on the penis are shown in Fig. 2f–h. The bursa copulatrix and the seminal receptacle are well developed. The former is rounded and large, while the latter is elongate and smaller. The seminal receptacle has a short duct that connects to the vagina, near the bursa. The vagina is long and straight, opening into the genital atrium. The vaginal gland is very well developed, elongate, flattened, with muscular walls and joins the vagina at the distal part.

Natural history. This species has been found in vertical rocky walls, mainly when *Tambja stegosauriformis* Pola et al., 2005a,b,c or *T. brasiliensis* sp. nov. (described

below) are abundant. It was observed that *R. ernsti* sp. nov. feeds on *T. stegosauriformis* (Fig. 1d) but probably also feeds on *T. brasiliensis* sp. nov.

Remarks. To date, there are eight species of *Roboastra* described: *Roboastra gracilis* (Bergh, 1877), *R. luteolineata* (Baba, 1936), *R. tigris* Farmer, 1978, *R. europaea* García-Gómez, 1985, *R. caboverdensis* Pola, Cervera and Gosliner, 2003, *R. leonis* Pola et al., 2005a,b,c; *R. tentaculata* (Pola et al., 2005a,b,c) and *R. ricei* Pola et al., 2005a,b,c. Of these species, none is found in Brazilian waters. In fact, the only species of the genus found in the waters of the Western Atlantic
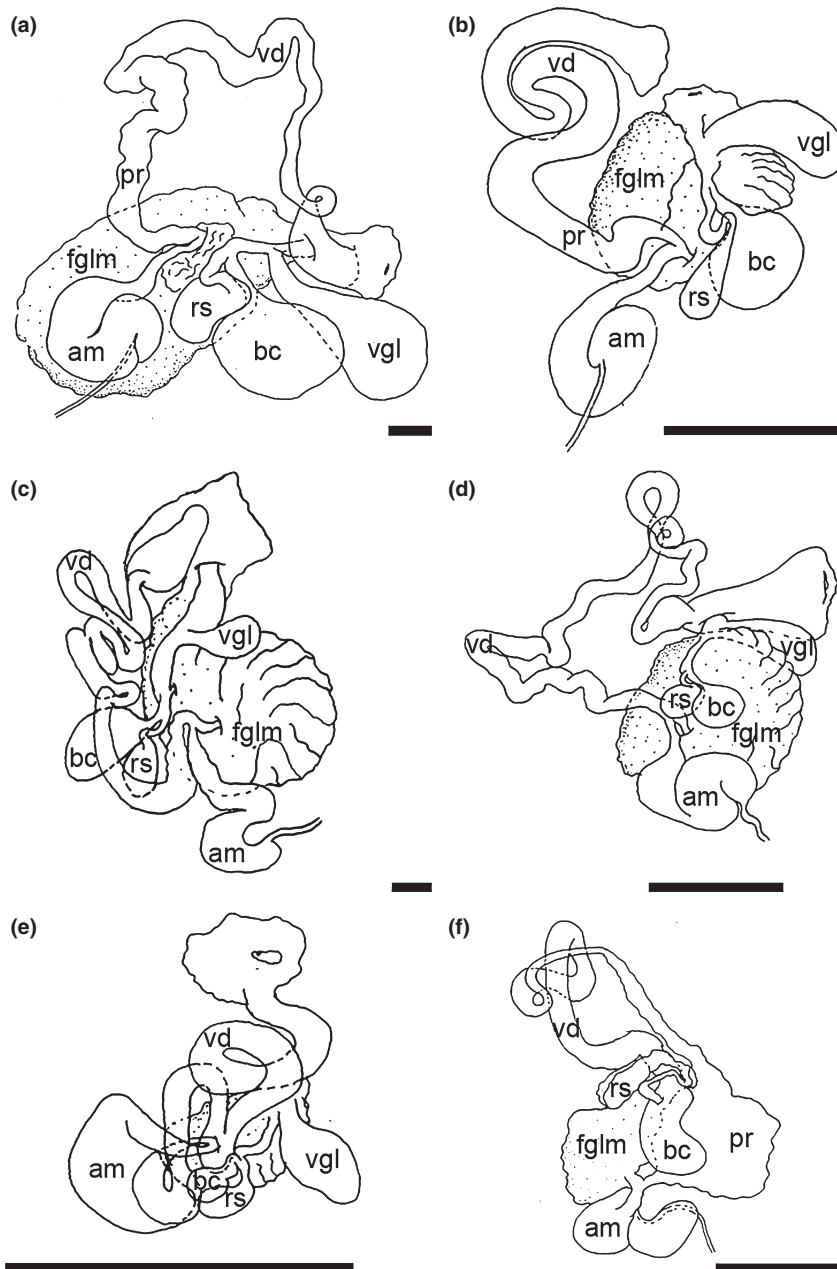
Fig. 3. Drawings of reproductive systems. (a) *Roboastra ernsti* sp. nov. (b) *Roboastra nikolasi* sp. nov. (c) *Tambja brasiliensis* sp. nov. (d) *Tambja crioula* sp. nov. (e) *Tambja kava* sp. nov. (f) *Tambja divae*. Scale bar: 1 mm.

is *R. ricei*, a very different species based on both external and internal morphology. Based on their colour pattern, the most similar species to *R. ernsti* sp. nov. are *R. leonis* and *R. luteolineata* but both are also very different and easy to distinguish. *Roboastra ernsti* lacks the two milky green markings between the rhinophores that make *R. luteolineata* unmistakable and *R. leonis* has a greater number of lines on the notum and both sides of the body, and the pigmentation of the gills, the rhinophores and most of the lines is different. Moreover, the morphology of the

teeth is also very different as in *R. leonis* the rachidian teeth have a small cusp in the middle and in *R. tigris* the middle cusps are longer and all the cusps are shorter and wider than in *R. ernsti* sp. nov. Besides, the inner laterals in *R. luteolineata* have a strongly curved bifid cusp (Pola et al., 2005a). It is remarkable that Ev. Marcus counted up to 100 rows of teeth, which is not common in this genus. We think this may be an error but we were not able to confirm it since the radula is missing. The DNA study carried out in this paper also confirmed that *R. ernsti* is a new

species of the genus and the second to be described from the Western Atlantic.

Roboastra nikolasi sp. nov. (Figs 1e–h, 3b, 4)

LSID. urn:lsid:zoobank.org:act:0F844410-2434-4F5D-834C-E4FD42796792.

Type material. Holotype: CASIZ 177031, Espiritu Santo Island, Bruat Channel, north coast of Malo Island (15°36.8′S, 167°08.5′E), Vanuatu archipelago, Pacific Ocean. 3 October 2006, 1 specimen, 15 mm alive (6 mm preserved), 10 m depth, collected by M. Pola and Y. Camacho. *Paratypes:* CASIZ 176778, Malaysia, South China Sea, Jubilee Shoal, 1 October 2007, 2 specimens, 13 m maximum depth, collected by T. M. Gosliner and D. W. Behrens.

Etymology. This species is dedicated to Nikolas Butvill-Camacho, first son of Yolanda Camacho-García, great friend to all the authors of this paper and who participated in the expedition Santo 2006 to Vanuatu, collecting and photographing this species.

Distribution. Thus far this species is known from Vanuatu (Gosliner et al., 2008 identified as *Roboastra* sp.; present study) and Malaysia (Coleman, 2008 [as *Tambja* sp.]; Gosliner et al., 2008 [as *Roboastra* sp.]; present study). It also seems to be present in Dampier, Western Australia (Coleman, 2001, 2008 [as *Tambja* sp.]), Japan (Coleman, 2008 as *Tambja* sp.) and Singapore (http://colorclouds.blogspot.com/2009_03_01_archive.html) but those specimens have not been studied and thus they could represent a different species.

External morphology (Fig. 1e–h). The body is elongate and limaciform with a long and pointed posterior end of the foot. The living animal is 15 mm in length. The body surface is wrinkled with several stripes slightly lighter on the notum and both sides of the body. The foot is linear at the posterior end. The anterior border of the foot shows well-developed lateral projections when the foot is extended (Fig. 1f). The head is rounded with a pair of conical, completely retractile, perfoliate rhinophores with about 15 lamellae. The oral tentacles are strongly developed and dorsolaterally grooved along part of their length (Fig. 1h). There are five small non-retractile tripinnate gill branches, all of them about the same size. The two lateral gill branches on each side are joined at their base. The gill forms a semicircle surrounding the anal papilla, which is elevated and is dark purple. The genital pore opens on the right side, midway between the gill and the rhinophores. The ground colour of this species is dark green with longitudinal lighter green or yellowish stripes. From each rhinophoral sheath a short, diffuse greyish black spot extends posteriorly in the dorsum. The posterior end of the body also has a diffuse greyish black spot. The oral region, including oral tentacles, and anterior border of the foot are dark blue/violet. Rhinophores are mainly dark blue/violet; the posterior lower third of their length is white; up to eight basal rhinophoral lamellae are white-pigmented in their posterior region; the upper four rhinophoral lamellae contain discrete whitish coloration on their internal areas. The gill branches are dark blue/violet externally; the inner side is white on the lower two-thirds of each branch length and dark blue/violet at the distal region. The dark blue edge of the foot connects to the oral region and to the dark spot at the posterior end of the body. Detailed study of the external and internal mantle shows some spicules. These specimens (except for a small piece of the foot) were originally preserved in formalin and thus the remaining spicules could have been dissolved by this preservative.

Internal morphology. The anterior digestive tract begins with a long, thick-walled muscular oral tube, which continues into a very small buccal bulb. At their junction, a pair of wide and elongate pouches opens into the digestive system (Fig. 4a,b). The salivary glands are short and thick, opening to the buccal mass and flanking the oesophagus. The labial cuticle is weak and lacks any armature. The radular formula is $32 \times 2(3).1.1.1.2(3)$ for both Vanuatu and Malaysia specimens. The rachidian tooth is rectangular, broad with a slightly arched upper edge and three well-developed denticles, the central one being slightly longer (Fig. 4c,d). The inner lateral tooth is very curved, having two very well-developed cusps. The inner cusp is bifid. All three cups are long, slender and shaped (Fig. 4c). There are only two outer lateral teeth, both lacking cups or denticulation. The inner outer lateral tooth is longer and larger than the outer laterals (Fig. 4c). There is a large, whitish and well-developed blood gland, granular in texture and which inserts into the dorsal surface of the oesophagus above the gut. The renal syrinx is visible under the pericardium, close to the anal papilla. After removing the entire body mass, at the very anterior edge of the foot, right after the mouth opening there is a very visible concentration of oral glands (Fig. 4e).

The reproductive system is triaulic (Fig. 3b). The hermaphroditic duct widens into a large S-shaped ampulla, which has thick walls. The ampulla narrows into the postampullary duct, which bifurcates into the vas deferens and oviduct. The short oviduct enters the female gland mass. The deferent duct lacks a morphologically well-differentiated prostate. It is long, wide and coiled. The penis is located within the distal end
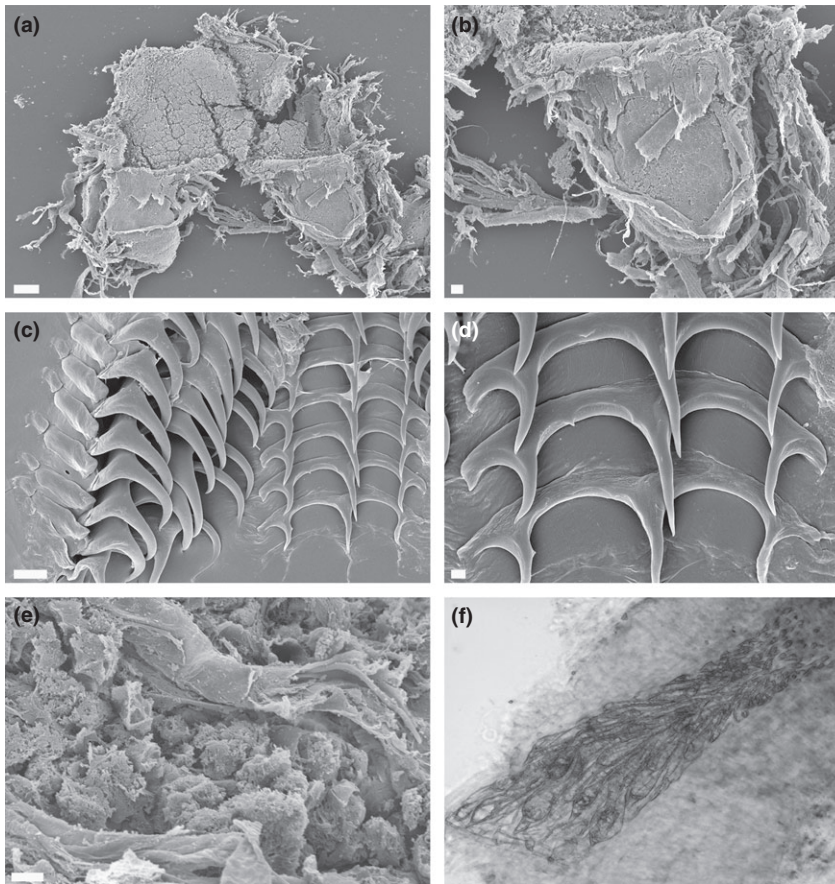
Fig. 4. *Roboastra nikolasi* sp. nov. (CASIZ 177031). (a, b) Elongate pouches. (a) Scale bar: 100 μm; (b) 20 μm. (c) Left half of the radula. Scale bar: 10 μm. (d) Rachidian teeth. Scale bar: 2 μm. (e) Penial spines. Scale bar: 400×. (f) Oral glands. Scale bar: 10 μm.

of the muscular deferent duct and it is armed with three different kinds of hooked and chitinous spines (Fig. 4f). The bursa copulatrix and the seminal receptacle are well developed. The bursa copulatrix is round and large. The seminal receptacle is smaller and much more elongate in shape than the bursa. It has a relatively long duct that connects to the vagina after making two loops. The vagina shares a common aperture within the genital atrium with the vaginal gland, which is very well developed, elongate, flattened, with muscular walls.

Natural history. Found on a sandy slope with *Halimeda* spp. and on arborescent bryozoans at 10–30 m depth.

Remarks. This species has been cited as *Tambja* sp. and *Roboastra* sp. in different books and webpages for several years (see *Distribution*). Externally, the strongly developed and dorsolaterally grooved oral tentacles indicate that it belongs to the genus *Roboastra*. Internally, the radula and penial spines confirm its identity. Species of *Roboastra* are usually larger and more robust, except for *R. gracilis* and *R. tentaculata*. Due to its size and colour pattern, *Roboastra nikolasi* sp. nov. cannot be confused with any other *Roboastra* described and it is clearly a new species, as confirmed by our molecular results. Note that there may be undescribed *Roboastra* species, more similar to *R. nikolasi* sp. nov. than other *Roboastra* species, such as those illustrated online (http://www.seaslugforum.net/showall/tambsp6) and identified as "*Tambja* sp.", "*Tambja* sp.6", "*Tambja oliva*", "*Tambja amakusana*?", and "*Tambja* cf. *amakusa*" (Koh, 2006), among others, but it is necessary to collect specimens to study their internal anatomy and obtain molecular data to confirm their identity.

Genus *Tambja* Burn, 1962

Type species. *Nembrotha verconis* Basedow *and* Hedley, 1905, by original designation.

Tambja brasiliensis sp. nov. (Figs 3c, 5a–d, 6)

LSID. urn:lsid:zoobank.org:act:3C406056-94F7-454A-83D9-B835751D5600.

Type material. *Holotype*: MNRJ 15123, Saco da Hípica, Ilha do Papagaio, Cabo Frio, Rio de Janeiro, Brazil, 13 December 2008, one specimen, 34 mm alive (17 mm preserved), 9 m depth, collected by V. Padula. *Paratypes*: MNRJ 15124, Saco da Hípica, Ilha do Papagaio, Cabo Frio, Rio de Janeiro, Brazil, 13 December 2008, one specimen, 28 mm alive (12 mm preserved), 9 m depth, collected by V. Padula. CASIZ 180375, Saco da Hípica, Ilha do Papagaio, Cabo Frio, Rio de Janeiro, Brazil, 13 December 2008, one specimen 32 mm alive (16 mm preserved), 9 m depth, collected by V. Padula. MZSP 92892, Segunda enseada, Ilha do Papagaio, Cabo Frio, Rio de Janeiro, Brazil, 13 December 2008, 3 specimens 15, 14 and 11 mm preserved, collected by V. Padula.

Additional material. MNRJ 15125, Saco do Poço, Ilhabela, São Paulo, Brazil, 3 December 2008, 1 specimen 23 mm alive (11 mm preserved), 6 m depth, collected by V. Padula. CASIZ 180374, Saco do Poço, Ilhabela, São Paulo, Brazil, 3 December 2008, 1 specimen 27 mm alive (10 mm preserved), 8 m depth, collected by V. Padula. MZSP 92890, Saco do Poço, Ilhabela, São Paulo, Brazil, 3 December 2008, 2 specimens 32 and 23 mm alive (12 and 9 mm preserved), 6 m depth, collected by V. Padula. MZSP 103242, Ilha de Serraria (23°48′68″S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 1 specimen 25 mm alive, max. depth 10 m, collected by V. Padula. MZSP 103243, Ilha de Serraria (23°48′68″S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 1 specimen 22 mm alive, max. depth 10 m, collected by V. Padula. MZSP 103244, Ilha de Serraria (23°48′68″ S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 1 specimen 30 mm alive, max. depth 10 m, collected by V. Padula. MZSP 103245, Ilha de Serraria (23°48′68″S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 1 specimen 20 mm alive, max. depth 10 m, collected by V. Padula. MZSP 103246, Ilha de Serraria (23°48′68″S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 1 specimen 23 mm alive, max. depth 10 m, collected by V. Padula. MZSP 103247, Ilha de Serraria (23°48′68″S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 7 specimens 20–30 mm alive, max. depth 10 m, collected by V. Padula, Y. Tibiriça and P. Oristanio. MZSP 103248, Ilha de Serraria (23°48′68″S, 45°13′72″W), São Paulo, Brazil, 20 January 2012, 9 specimens 20–35 mm alive, max. depth 10 m, collected by V. Padula, Y. Tibiriça, P. Oristânio and J.C. García.

Etymology. In reference to the species type-locality and geographical distribution in Brazil, and its main colours (yellow, green and blue) that match the colours of the Brazilian flag.

Distribution. This species is known from south-eastern and southern Brazil, from Rio de Janeiro to Santa Catarina states (present study; Debelius and Kuiter, 2007 [identified as *Tambja* sp.12]).

External morphology (Fig. 5a–d). The body is small, elongate and limaciform with a long and pointed posterior end of the foot. The head is rounded. The living animals reach 35 mm in total length with the body surface noticeably wrinkled. There is a pair of large and conical, completely retractile, perfoliate rhinophores with approximately 20 tightly packed lamellae. The rhinophoral sheaths are well developed. The oral tentacles are short, dorso-ventrally flattened and horizontally grooved. There is a distinctive translucent dark patch behind each rhinophore above where the eye is situated on the nerve ring. There are five non-retractile tripinnate gill branches forming a semicircle around the tubular anal papilla. The two posteriormost branches of each side share a common base. The genital pore opens on the right side, between the gill and rhinophores, closer to the gill. Small lateral slots of unknown function located below the rhinophores found in other species of *Tambja* (Yonow, 1994; Pola et al., 2005b,c, 2006b) are also present in this species (Fig. 5c). Detailed study of the external and internal mantle also shows the presence of numerous spicules (Fig. 5d). These spicules are simple and arranged on both sides of the body from behind the oral tentacles to close to the gill circle. These specimens (except for a small piece of the foot) were originally frozen and later moved to 70% EtOH, and thus the spicules have been preserved. The ground colour is yellow, ranging from light orange to light green, and covered with numerous small bright blue spots scattered on the notum and both sides of the body. The edge of the foot varies from light green to dark blue. The posterior end of the foot is dark blue. The oral tentacles are largely dark blue, but are light green near to the mouth. The areas above the eyes, behind the rhinophores, have a darker colour than the rest of the mantle. The bottom half of the rhinophores is dark green and the distal portion is blue. Externally, the base of each gill branch is the same colour as the body, followed by a short white area and a blue apex. Internally the rachis and most areas of the leaves are opaque white with a blue apex.

Internal morphology. The anterior digestive tract begins with a short, thick-walled muscular oral tube
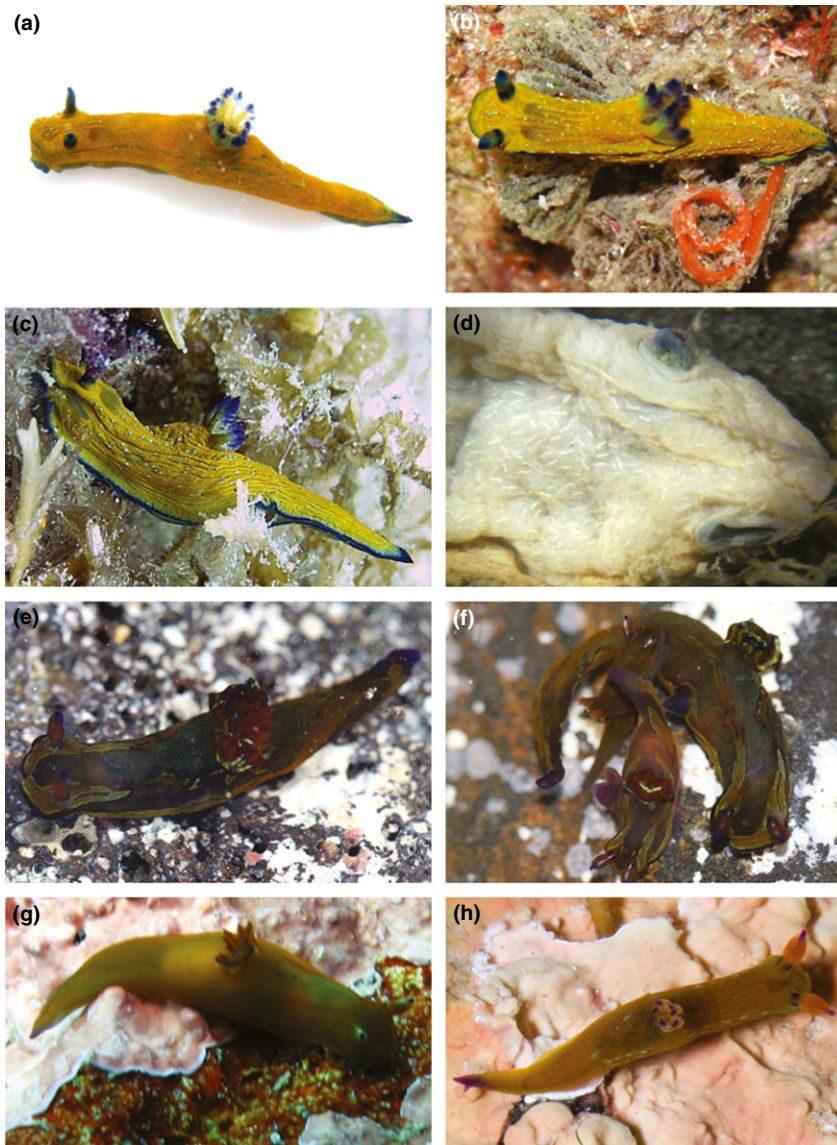
Fig. 5. Photographs of the living animals. (a–d) *Tambja brasiliensis* sp. nov., Brazil. (a) MZSP 103242, 25 mm in length, São Paulo (photo: Vinicius Padula). (b) MZSP 92890, 23 mm in length, São Paulo (photo: Vinicius Padula). (c) Specimen showing lateral slots (photo taken from Picassa, Klaas, 2008). (d) Detail of the spicules in the mantle (MZRJ 15123) (photo by Vinicius Padula). (e, f) *Tambja crioula* sp. nov., Cape Verde Archipelago. (e) MNCN15.05/60097 (Paratype), 35 mm in length, Ilheu dos Pássaros (photo: Marta Pola). (f) Holotype and paratypes, 35, 25 and 15 mm in length, Ilheu dos Pássaros (photo: Marta Pola). (g) CASIZ 180377, 20 mm in length, Ilha de Santiago (photo: Peter Wirtz). (h) *Tambja kava* sp. nov., Vanuatu. CASIZ 178792, 15 mm in length, Western Aoré Island (photo: Yolanda Camacho).

that continues into a medium-sized, muscular buccal mass. There is a pair of large, wide salivary glands on the buccal mass, flanking the oesophagus. The labial cuticle forms a strong chitinous disc, without distinct armature but with folds in its internal edge (Fig. 6a). The radular formulae of six specimens are: 16 × 5.1.1.1.5 (MNRJ 15123, holotype), 16 × 5.1.1.1.5 (CASIZ 180374, Fig. 6b), 17 × 5.1.1.1.5 (CASIZ 180375) and 18 × 5.1.1.1.5 (MZSP 103243, MZSP 103244 and MZSP 103246). The rachidian teeth are broad and rectangular, without denticles and smooth at the upper margin, which is slightly curved (Fig. 6b,c). The inner lateral teeth are much larger than the outer ones, with two well-developed cusps; the inner cusp is bifid, with two sharp denticles. The inner edge of the upper cusp is slightly serrated (Fig. 6c,d). The outer laterals are rectangular plates, without cusps or dentition, becoming smaller near the margin (Fig. 6b–d). There is a large, whitish, well-developed blood gland, which is granular in texture and inserts into the dorsal surface of the oesophagus above the gut. The renal syrinx is visible under the
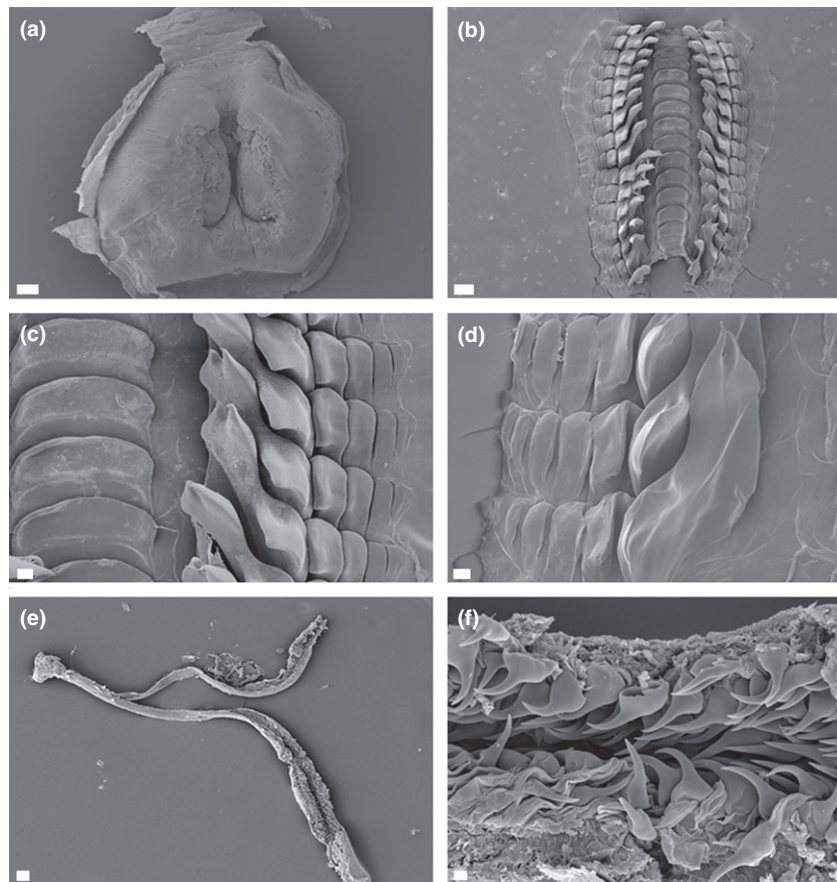
Fig. 6. Scanning electron micrographs of *Tambja brasiliensis* sp. nov. (a) Labial cuticle (CASIZ 180375). Scale bar: 100 μm. (b) Radula (CASIZ 180374). Scale bar: 100 μm. (c) Right half of the radula (CASIZ 180374). Scale bar: 20 μm. (d) Left half of the radula (CASIZ 180375). Scale bar: 20 μm. (e) Penis (CASIZ 180375). Scale bar: 30 μm. (f) Penial spines (CASIZ 180375). Scale bar: 3 μm.

pericardium, close to the anal papilla. After removing the entire body mass, at the very anterior edge of the foot, immediately below the mouth opening and in the surrounding areas there is a highly visible concentration of oral glands.

The reproductive system is triaulic (Fig. 3c). The preampullatory duct is elongate and narrow. It expands into a thick-walled, "S"-shaped ampulla, which divides into the oviduct and the vas deferens. From this point the oviduct enters the massive female gland via a short duct. The deferent duct is long and coiled over it. The deferent duct is about the same diameter along its entire length. It lacks a morphologically well-differentiated prostate. Within the distal end of the muscular portion of the vas deferens is located the penial bulb. The penis is armed with small, hooked and chitinous spines (Fig. 6e,f). The seminal receptacle is smaller and more elongate than the globular bursa copulatrix, with a short duct that connects to the vagina after making two loops, midway to the bursa. A slender uterine duct leaves the vagina and joins with the female gland. A well-developed vaginal gland is present.

Natural history. Found on vertical walls covered with *Bugula* spp. between 5 and 30 m depth. *Tambja brasiliensis* lays a spiral egg mass with around two whorls and many small orange eggs (Fig. 5b), around 25 rows from the base to the free edge of the egg mass. It seems that later in development the embryos turn white, as we observed in some egg masses in the field. *Tambja brasiliensis* contains the chemical compounds tambjamine A and D (Granato et al., 2005; as "*Tambja eliora*") obtained from its prey, the bryozoan *Bugula dentata* (Pereira et al., 2012). In experiments, tambjamine D showed cytotoxicity against different lineages of human cancer cells (Granato et al., 2005).

Remarks. To date, only two species of *Tambja* have been described from Brazil: *Tambja divae* (Marcus, 1958) and *T. stegosauriformis*. *Tambja stegosauriformis* is very easy to distinguish as its colour is very characteristic and it has prominent tubercles forming a crest behind the gill ending on the tip of the tail. *Tambja divae* also shows several external and internal differences from *T. brasiliensis*

sp. nov. *Tambja divae* is bright orange with many small white dots and white gills while *T. brasiliensis* sp. nov. ranges from yellow to light orange or light green with many bright blue dots, and the gill is externally yellow with a blue apex. Externally, other important differences are the presence of a posterior median crest in *T. divae*, not present in *T. brasiliensis* sp. nov., and the presence of three gill leaves in *T. divae* instead of five in *T. brasiliensis* sp. nov. There are also two important internal differences. In *T. divae* the inner lateral teeth are long hooks with a strong cusp and irregular processes between cusp and base, and a vaginal gland is absent in the reproductive system (Marcus, 1958; present study), whereas in *T. brasiliensis* sp. nov. the inner lateral teeth have two well-developed cusps and the inner tooth is bifid with two sharp denticles, and also a vaginal gland is present. Debelius and Kuiter (2007) show two pictures identified as "*Tambja* sp.12" and comment that they could be *T. divae*. However, based on the differences described above, which are consistent in more than 30 specimens and the fact that *T. divae* has not been found since its original description, we describe here *Tambja brasiliensis* sp. nov. as the third species of the genus known only from Brazil.

Tambja crioula sp. nov. (Figs 3d, 5e–g, 7)

LSID. urn:lsid:zoobank.org:act:111FA744-D5EB-4016-95BD-8B551A5FB20A.

Type material. Holotype: MNCN 15.05/60096. Ilheu dos Pássaros (16°54′36.27″N, 25°0′43.67″W), Mindelo, São Vicente, Cape Verde, 13 July 2013, 1 adult specimen 25 mm alive (15 mm preserved), 14 m depth, not dissected, collected by M. Pola. Paratypes: MNCN 15.05/60097. Ilheu dos Pássaros (16°54′36.27″N, 25°0′43.67″W), Mindelo, São Vicente, Cape Verde, 13 July 2013, 1 adult specimen 35 mm alive (21 mm preserved), 14 m depth, dissected, collected by M. Pola. MNCN 15.05/60098. Ilheu dos Pássaros (16°54′36.27″N, 25° 0′43.67″W), Mindelo, São Vicente, Cape Verde, 13 July 2013, 1 adult specimen 15 mm alive (10 mm preserved), 14 m depth, dissected, collected by M. Pola. CASIZ 180377, Tarrafal, Ilha de Santiago, Cape Verde Archipelago, 10 July 2008, 1 specimen, 20 mm alive (12 mm preserved), 8 m depth, dissected, collected by P. Wirtz.

Etymology. In reference to Cape Verde creole, a language from the Cape Verde Archipelago.

Distribution. Thus far, this species is only known from Cape Verde.

External morphology (Fig. 5e–g). The body is elongate and limaciform with a long and pointed posterior end of the foot. The head is rounded with the frontal veil reduced to a visible pallial rim. The living animal reaches 35 mm in total length with the body surface smooth. There is a pair of small and conical, completely retractile, perfoliate rhinophores with approximately 15–20 tightly packed lamellae. The rhinophoral sheaths are well developed. The oral tentacles are short, dorso-ventrally flattened and horizontally grooved. There are five non-retractile tripinnate gill branches forming a semicircle around the anal papilla. The two lateral branches of each side share a common base. All the gill branches are about the same size. The genital pore opens on the right side, midway between the gill and rhinophores. The lateral slots of unknown function located below the rhinophores found in other species of *Tambja* are also present in this species. Detailed study of the external and internal mantle shows the presence of simple spicules scattered on both lateral sides of the body. The spicules are also found on the foot. These specimens were originally preserved in 98% EtOH. The ground colour is olive green. There are three pairs of longitudinal yellow green bands that run parallel from the head to the tail, never joining at the posterior end. The first band follows the notal edge. It continues along the edge of the head, surrounding the rhinophores and ends posteriorly to the gill. The second band is located on the flanks, from under the upper edge of the oral tentacles to the tail, surrounding the genital opening and ending posteriorly to the notal edge band. The third band borders the whole edge of the foot to the end of the tail. All these lines or bands may be interrupted, giving a sense of apparent intraspecific variability. Moreover, in the smaller specimens, these bands are emerging and the pattern is not clear. These specimens seem to be entirely olive with a thin pair of purple lines. The lamellae of the rhinophores are mostly vermilion but the lower lamellae are purple and those of the tips are blue. There is also a white pigmentation along the median posterior line of the rhinophore. The oral tentacles, the edge of the foot, the end of the tail and the genital opening are purple. The branchial leaves are vermilion, with the lower part of the outer rachis olive and the upper part lime. The inner rachis is entirely lime.

Internal morphology. The anterior digestive tract begins with a short oral tube that continues into a large, muscular buccal mass. At their junction, a pair of small and elongate pouches opens into the digestive system (Fig. 7a). There is a pair of large, wide, granular salivary glands on the buccal mass, flanking the oesophagus. The labial cuticle is thick and
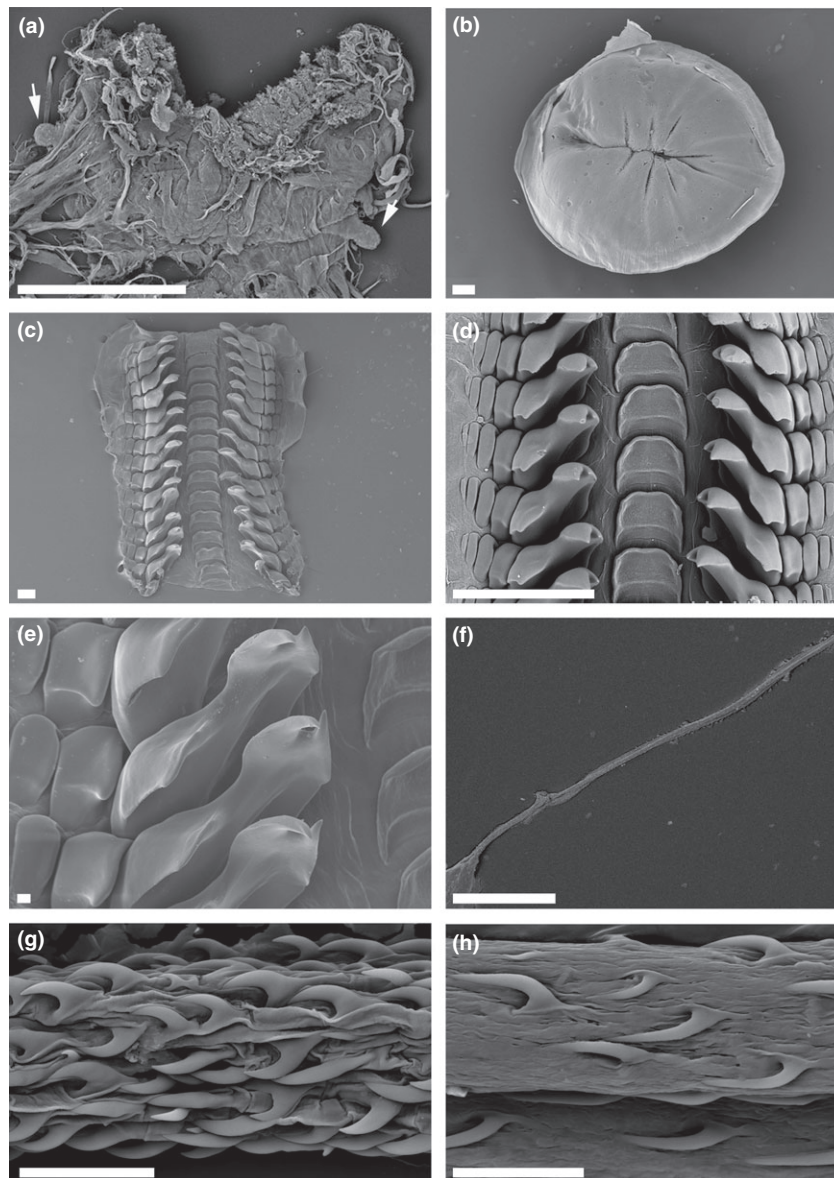
Fig. 7. Scanning electron micrographs of *Tambja crioula* sp. nov. (a) Elongate pouches (MNCN15.05/60098). Scale bar: 1 mm. (b) Labial cuticle (CASIZ 180377). Scale bar: 100 μm. (c) Radula (CASIZ 180377). Scale bar: 100 μm. (d) Half radula (MNCN15.05/60098). Scale bar: 300 μm. (e) Detail of the innermost lateral teeth (CASIZ 180377). Scale bar: 20 μm. (f) Penis (MNCN15.05/60097). Scale bar: 500 μm. (g) Basal penial spines (MNCN15.05/60097). Scale bar: 20 μm. (h) Distal penial spines (MNCN15.05/60097). Scale bar: 20 μm.

chitinous, lacking denticles (Fig. 7b). The radular formulae of the three specimens dissected are 18 × 4.1.1.1.4 (MNCN 15.05/60097, 35 mm long), 16 × 4.1.1.1.4 (CASIZ 180377, 20 mm long) and 15 × 4.1.1.1.4 (MNCN 15.05/60098, 15 mm long) (Fig. 7c). The rachidian teeth are broad, without denticles (Fig. 7c,d). The inner lateral teeth are much larger than outer ones, with two well-developed cusps: the inner cusp is bifid, with two sharp and elongate denticles, the outer one slightly longer (Fig. 7c–e); the outer cusp is smaller and rectangular in shape. The outer lateral teeth are plate-like, simple and decrease

in size towards the outer margin (Fig. 7d). There is a whitish, well-developed blood gland, which is granular in texture and which inserts into the dorsal surface of the oesophagus above the gut. The renal syrinx is visible under the pericardium, close to the anal papilla.

The reproductive system is triaulic (Fig. 3d). The preampullatory duct is elongate and narrow. It expands into a large thick-walled, "S"-shaped ampulla, which divides into the oviduct and the vas deferens. From this point the oviduct enters the massive female gland via a short duct. The vas deferens is very long and highly coiled, slightly narrower toward the penial

bulb. It lacks a morphologically well-differentiated prostate. The prostate is recognizable by having slightly soft walls and slightly wider diameter. Within the distal end of the muscular portion of the vas deferens the penial bulb is located. The penis is armed with hooked and chitinous spines, which are more abundant and shorter closer to the vas deferens and less numerous and larger at the distal end of the penis (Fig. 7f–h). The vaginal duct connects to the round bursa copulatrix. The seminal receptacle is large and pyriform. A duct joins the seminal receptacle to the vagina after completing two loops. The vaginal gland is large, thick-walled and well developed. After removing the entire body mass, at the very anterior edge of the foot, right below the mouth opening and in the surrounding lateral areas there is a very visible concentration of oral glands.

Remarks. *Tambja crioula* sp. nov. is the fifth species of the genus (*T. fantasmalis* Ortea and García-Gómez, 1986; *T. ceutae* García-Gómez and Ortea, 1988, *T. anayana* Ortea, 1989 and *T. simplex* Ortea and Moro, 1998) present in the Cape Verde Archipelago. The juvenile specimens of *Tambja crioula* sp. nov. resemble *T. anayana* Ortea, 1989 but there are external and internal morphological as well as anatomical differences that allow us to distinguish these species. *T. anayana* is known only on the basis of a single 6-mm specimen from Boavista Island. Also, the original description of *T. anayana* lacks pictures of the live animal and the reproductive system is not described. Thus, we compare the new species with the information available in Ortea (1989).

Ortea (1989) described his specimen of *T. anayana* as light khaki green with two purple lines along the mantle, joining anteriorly in front of the rhinophores, and posteriorly behind the gill, running as a single line until the end of the tail. This colour pattern could be confused with the coloration of our youngest specimens, but in addition to that in our specimens the lines never join. Ortea (1989) also described some scattered black dots, which are missing in *Tambja crioula*. The author also mentioned and drew a violet line between the rhinophores as well as two violet short lines on both sides of the head, in the first quarter somewhat behind the rhinophores (see Ortea, 1989; Fig. 1a, p. 32). This is not the pattern of lines shown in *T. crioula* sp. nov. Other external differences are: *T. anayana* has three gill branches while the new species has five and the number of lamellae in rhinophores are seven in *T. anayana* and 15–20 in *T. crioula* sp. nov. Regarding the internal anatomy, there are no spicules, salivary glands, blood gland or reproductive system described for *T. anayana* so is not possible to compare any of these features with our specimen. However, the radula is available for comparison. Even

though Ortea (1989) did not give a detailed description of each tooth, he drew a few of them. Thus, the rachidian teeth of *T. anayana* are more or less quadrangular while in *T. crioula* sp. nov. they are clearly rectangular. Regarding the inner laterals, Ortea explained that in *T. anayana* the inner laterals from row 1 to 22 are bifid, with a progressive reduction of the size of the second cusp, the last inner laterals having only one cusp. Looking at our specimen, all the inner laterals have an upper bifid cusp, with the second cusp well developed even in the last rows. *T. fantasmalis* and *T. simplex* can be clearly distinguished from *T. crioula* sp. nov. by their colour pattern. The ground colour of *Tambja fantasmalis* is dark blue, almost black with two light green bands on the notum never joining posteriorly (Ortea and García-Gómez, 1986; Pola et al., 2006a,b,c). In *T. simplex* the ground colour is black-purple and has the edge of the notum and foot yellow and the notum with a mid-yellow line (Ortea and Moro, 1998; Cervera et al., 2000; Pola et al., 2006a,b,c). Internally, *T. fantasmalis* and *T. simplex* are also very similar to the new species. The radular formula and the shape of the teeth are almost identical and the reproductive system is quite similar but the penial spines of *T. simplex* are much more numerous and elongate thanin *T. fantasmalis* and *T. crioula* sp. nov. (Pola et al., 2006a,b,c). However, the elongated pouches opening into the oral tube have never been observed in *T. fantasmalis* or *T. simplex*. In our molecular results, *T. crioula* sp. nov. is closed related to *T. fantasmalis* and these two species form a very well-supported clade with *T. simplex* and *T. marbellensis* Schick and Cervera, 1998 (Figs 12 and 13).

Tambja kava sp. nov. (Figs 3e, 5h, 8a–d)

LSID. urn:lsid:zoobank.org:act:9B1FECE0-A2D1-485A-95B7-26AED06538AE.

Type material. Holotype: CASIZ 178792, Western Aoré Island (15°33.1′S, 167°09.6′E), Vanuatu Archipelago, 8 October 2006, 1 specimen 15 mm alive (4 mm preserved), 9–31 m depth, collected by M. Pola and Y. Camacho.

Etymology. The specific name "*kava*" refers to the most popular drink consumed in Vanuatu, where this species was collected, as well as throughout the western Pacific Ocean cultures of Melanesia.

Distribution. The confirmed distribution of this species is the Vanuatu Archipelago (present study).

External morphology (Fig. 5h). The body is very small, elongate and limaciform with a long and

pointed posterior end of the foot. In the only available specimen, the posterior end of the foot is bifid but this is most likely a teratology. The ground colour is uniformly greenish with whitish markings arranged along the mantle edge that join behind the circular gill. These markings appear as a white broken line along the mantle edge. There are numerous longitudinal reddish stripes that give the body a corrugated appearance. There is a pair of delicate, perfoliate rhinophores with about 16 lamellae, which are retractile in their sheaths. The oral tentacles are thin flat lobes. Five short, tripinnate non-retractile gill branches form a semicircle around the anal papilla. The tips, upper lamellae and bases of the rhinophores, the oral tentacles, the upper half of the branchial plumes and the posterior end of the foot are purple. The remaining rhinophoral lamellae are reddish. The basal outer and inner branchial rachises are white. The rhinophoral sheaths are the same colour as the body. The genital pore opens on the right side, midway between the gill and the rhinophores. Small lateral

slots are present on both sides of the body, between the rhinophores and the oral tentacles. Detailed study of the external and internal mantle does not show clearly the presence of spicules on the mantle, although tiny fragments of them may be present. This specimen (except for a small piece of the foot) was originally preserved in formalin and thus there is a chance that the calcium carbonate spicules could have been dissolved by this acidic preservative.

Internal morphology. The anterior digestive tract begins with a very short oral tube that continues into a large buccal mass. There is a pair of large, wide, granular salivary glands on the buccal mass, flanking the oesophagus. The labial cuticle is thick and chitinous, lacking denticles. The radular formula of the holotype is $15 \times 4.1.1.1.4$ (CASIZ 178792) (Fig. 8a). The rachidian teeth are rectangular, without denticles and have a smooth upper edge (Fig. 8a,b). The inner lateral teeth are much larger than the outer ones, with two well-developed cusps: the upper cusp is
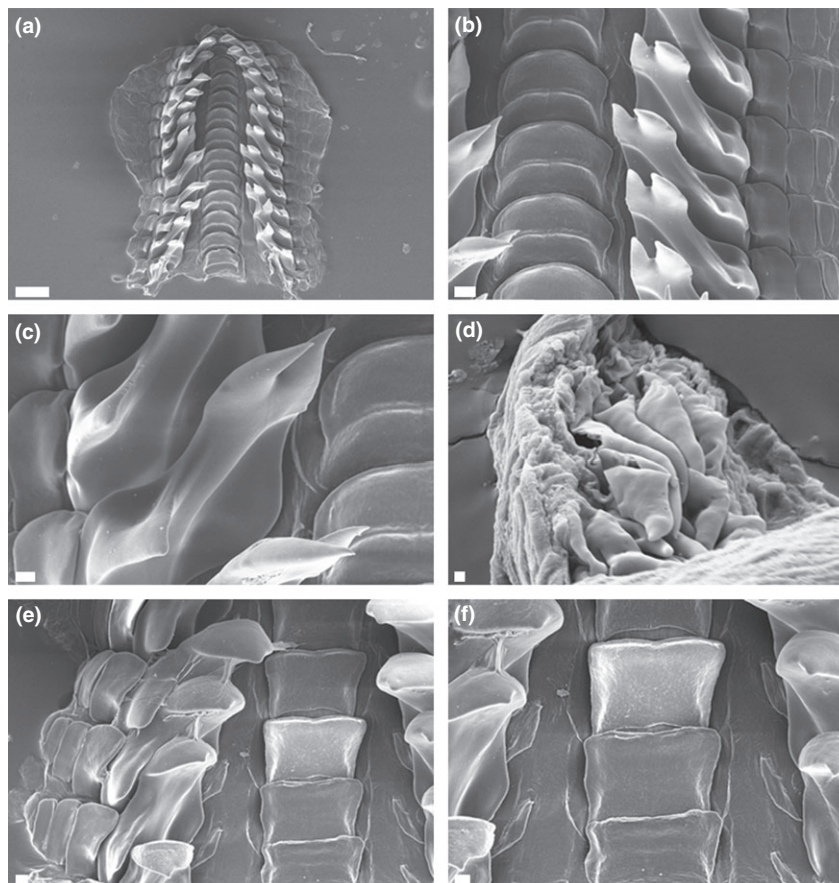
Fig. 8. Scanning electron micrographs of *Tambja kava* sp. nov. and *Tambja* cf. *amakusana*. (a–d) *Tambja kava* sp. nov. (CASIZ 178792, Holotype), Vanuatu. (a) Radula. Scale bar: 100 μm. (b) Right half of the radula. Scale bar: 20 μm. (c) Innermost lateral tooth. Scale bar: 10 μm. (d) Penial spines. Scale bar: 1 μm. (e, f) *Tambja* cf. *amakusana* (CASIZ 179284), Vanuatu. (e) Left half of the radula. Scale bar: 10 μm. (f) Rachidian teeth and vestigial teeth. Scale bar: 10 μm.

bifid, with two sharp and elongate denticles, both about the same size (Fig. 8b,c); the outer cusp is smaller and rectangular in shape. The outer lateral teeth are plate-like, simple and decrease in size towards the outer margin (Fig. 8a,b). The blood gland is granular and well developed. It inserts into the dorsal surface of the oesophagus above the gut. The renal syrinx is visible under the pericardium, close to the anal papilla. The guts of this specimen were full of bryozoans. After removing the entire body mass, at the very anterior edge of the foot, right after the mouth opening there is a very visible concentration of oral glands.

The reproductive system is triaulic (Fig. 3e). The hermaphroditic duct widens into a very large S-shaped thick-walled ampulla. The ampulla narrows into the postampullary duct, which bifurcates into the vas deferens and the oviduct. The short oviduct enters the female gland mass, which is well developed. The deferent duct lacks a morphologically well-differentiated prostate. It is long, wide and coiled. The penis is located within the distal end of the muscular deferent duct and it is armed with small, hooked and chitinous spines (Fig. 8d). The bursa copulatrix and the seminal receptacle are well developed. The bursa copulatrix is round and slightly smaller than the seminal receptacle, which is more elongate in shape than the bursa. The seminal receptacle has a relatively wide, straight duct that connects to the vagina. The vagina shares a common aperture within the genital atrium with the vaginal gland, which is very well developed, elongate, flattened, with muscular walls.

Remarks. The only known species of the genus that resembles our specimen is *Tambja amakusana* Baba, 1987; described from Japan from a single living specimen 8 mm in length. The description of Baba (1987) and the subsequent description by Marshall and Willan (1999) were later expanded by Pola et al. (2006b). *Tambja amakusana* has several remarkable features that allow it to be distinguished from our new species, based mostly on internal anatomy. For instance, *T. amakusana* has elongate pouches at the junction of the oral tube and the buccal bulb, which are apparently absent in *T. kava* sp. nov. The radula is also quite different. In *T. amakusana* the rachidian teeth (Fig. 8e) are nearly quadrangular in shape and the inner laterals have an upper bifid cusp with a wide and blunt terminal denticle and a very small inner one. However, in our specimen of *T. kava* sp. nov., the rachidians (Fig. 8a,b) are rectangular and the inner lateral teeth have a deeply bifid upper cusp with two sharp and elongate denticles, both about the same size. Two other major differences in the radula are the number of marginals (five vs. four in *T. amakusana* and *T. kava* sp. nov., respectively) and a small

projection, between the rachidian tooth and the inner lateral tooth, described as "vestigial tooth" for *T. amakusana* (Pola et al., 2006b) and is not present in the new species. In addition, the reproductive system of *T. amakusana* is also very different from that of *T. kava* sp. nov., as it has a prostatic portion with a dense network of interconnecting tubules over its surface and it lacks a vaginal gland, among other differences. In *T. kava* sp. nov. the deferent duct lacks a morphologically well-differentiated prostate and there is a very well-developed and elongate vaginal gland. Lastly, our molecular analysis recovered *T. kava* sp. nov. within a completely different clade from that which includes *T. amakusana*, *T. limaciformis* and *T. divae*. *Tambja kava* sp. nov. appears to be more closely related to *T. brasiliensis* sp. nov. and *Tambja* cf. *tenuilineata*, from Mexico. The specimen of *T. kava* sp. nov. studied here was previously misidentified as *T. amakusana* by Gosliner et al. (2008). The specimens photographed and identified as *T. amakusana* by C. Y. Wong (http://www.flickr.com/photos/cywong/3614313252/) and *Roboastra* sp. by J. Davies from Indonesia (http://www.nudipixel.net/species/roboastra_sp/) also seem to belong to the same species as our specimen, but they have not been study anatomically and thus their identity is not yet confirmed.

Tambja cf. *amakusana* Baba, 1987 (Figs 8e–f, 9a)

Material examined. CASIZ 179284, Western Tutuba Island, Vanuatu Archipelago, Pacific Ocean (15°33.6′S, 167°16.5′E). 9 October 2006, 1 juvenile specimen 10 mm alive (2 mm preserved), 33 m depth, collected by M. Pola and Y. Camacho on large mound with rubble, sand and scattered corals.

External morphology (Fig. 9a). The body is very small, elongate and limaciform with a long and pointed posterior end of the foot. The ground colour is translucent white, with some areas covered by a light orange pigment. The eyes are visible through the skin. There is a pair of delicate perfoliate rhinophores with about 10 lamellae, which are retractile in their sheaths. The oral tentacles are thin flat lobes. Three very small, bi-pinnate non-retractile gill branches form a semicircle around the anal papilla. The rhinophores have anterior and posterior orange regions. The lateral and apical areas are white and the tips are dark violet. Some basal lamellae also have dark violet spots.

The gill branches are white with a dark violet apex. The genital pore opens on the right side, midway between the gill and the rhinophores. Small lateral slots are present on both sides of the body, between the rhinophores and the oral tentacles. Detailed study

of the external and internal mantle does not indicate the presence of spicules. This specimen was entirely preserved in formalin and thus it is possible that the calcium carbonate spicules could have been dissolved by this acidic preservative.

Internal morphology. The anterior digestive tract begins with a very short oral tube that continues into the wide, muscular buccal mass, which is relatively large in comparison with the size of the animal. There is a pair of large, wide and granular salivary glands on the buccal mass, flanking the oesophagus. The labial cuticle is thick and chitinous, lacking denticles. The radular formula of the single studied specimen is $10 \times 4.1.1.1.4$ (CASIZ 179284). The rachidian teeth are broad, nearly quadrangular, without denticles but with a notched upper edge (Fig. 8e). The inner lateral teeth are much larger than the outer ones, with two well-developed cusps: the upper cusp is bifid, with a wide and blunt terminal denticle and a very small inner one (Fig. 8e, f); the outer cusp is shorter. The outer lateral teeth are plate-like, decreasing in size towards the outer margin (Fig. 8e). There is a vestigial tooth between the rachidian and the inner lateral teeth. After removing the entire body mass, at the very anterior edge of the foot, right after the mouth opening there is a very visible concentration of oral glands. The reproductive system of this specimen is not developed.

Remarks. This immature specimen matches the original description of *T. amakusana* concerning external morphology, colour and radular characteristics. These features were previously discussed in the remarks for *T. kava*, suggesting that the differences between these two species appear to be present independent of specimen size. We prefer not to confirm the identity of this small specimen as no data on the reproductive system and no molecular sequences could be obtained.

Tambja divae (Marcus, 1958) (Figs 3f, 9b–c, 10)

Type material. Holotype: MZSP 76055, Praia do Forno, Arraial do Cabo (previously part of Cabo Frio city), Rio de Janeiro State, Brazil, 15 July 1957, under a stone in the tidal zone, collected by Diva Diniz Corrêa (Marcus, 1958).

Additional material. MZSP 103230, Parcel da pedra lisa, Ilha de Buzios (23°47′45″S, 45°08′69″W), São Paulo, Brazil, 19 January 2012, 1 mature specimen 11 mm alive, 6 m depth, under rocks, collected by C. Cunha.

Distribution. Thus far, this species is only known from south-eastern Brazil, from Rio de Janeiro and São Paulo states (Marcus, 1958; present study).

External morphology. (Fig. 9b–c). The body is very small, elongate and limaciform with a long and pointed posterior end of the foot. The body surface is quite smooth or slightly wrinkled. The head is rounded but with a slightly bilobed frontal veil. There is a pair of stout, conical, completely retractile, perfoliate rhinophores with approximately 17 tightly packed lamellae. To the sides of the rhinophores, a pair of low crests runs posteriorly separating the notum from the sides. These crests join behind the gills, resulting in a posterior median low crest that ends on the tip of the foot. The oral tentacles are short. There are three non-retractile tripinnate gill branches forming a semicircle around the anus. The three gill-branches are very similar in size. The genital pore opens on the right side, between the gill and rhinophores, closer to the gill. Detailed study of the external and internal mantle also shows the presence of spicules. The ground colour is vivid orange with white tips to the gills and white dots scattered on the notum. The tips of the rhinophores are also white but the lamellae and the rest of the body is uniform in colour.

Internal morphology. The anterior digestive tract begins with a very short oral tube that continues into a large buccal mass, relatively large compared with the overall body size. At their junction, a pair of large elongate pouches opens into the digestive system (Fig. 10a,b). There is a pair of small, narrow, granular salivary glands on the buccal mass, flanking the oesophagus. The labial cuticle is smooth. The radular formula of the holotype is $16 \times 5{-}6.1.1.1.5{-}6$ (Marcus, 1958) while the specimen studied here has a formula of $12 \times 6.1.1.1.6$ (Fig. 10c). The rachidian teeth are quite flat plates, almost quadrangular, without denticles. Their anterior edge is not recurved although they seem to have a very slight notch in the middle (Fig. 10c,d). The inner lateral teeth are long hooks with a strong upper cusp: the upper cusp has a prominent protrusion (Fig. 10c,d). The outer lateral teeth are plate-like, simple and decrease in size towards the outer margin (Fig. 10c,d). The blood gland is granular and well developed. It inserts into the dorsal surface of the oesophagus above the gut. The renal syrinx is visible under the pericardium, close to the anus.

The reproductive system is triaulic (Fig. 3f). The hermaphroditic duct widens into a very large S-shaped thick-walled ampulla. The ampulla narrows into the postampullary duct, which bifurcates into the vas deferens and the oviduct. The short oviduct enters the female gland mass, which is well developed. The

Fig. 9. Photographs of the living animals. (a) *Tambja* cf. *amakusana* (CASIZ 179284), 10 mm in length, Vanuatu (photos: Yolanda Camacho). (b, c) *Tambja divae*, Brazil. (b) MZSP 76055 (parts of the holotype). (c) MZSP 103230, 11 mm in length (photos: Vinicius Padula). (d) *Tambja marbellensis* (CASIZ 180379), Portugal (photo: Fatima Mar). (e) *Tambja zulu* showing very well-developed oral tentacles and lateral slots, Durban (photo: Valda Fraser). (f) *Roboastra gracilis* crowling on bryozoans, Malaysia (photo: Kheong Chan). (g) *Roboastra gracilis* "feeding?" on bryozoans, Queensland (photo: Paul Osmond). (h) *Roboastra tentaculata* feeding on blue bryozoans (photo: Esperanza Manzano).

branch of the vas deferens widens into a large, curved prostatic portion, having a dense network of interconnecting tubules over its surface. The vas deferens again narrows into a thin duct, which descends through the centre of the large, highly convoluted terminal portion of the duct. *In situ*, the bursa is partially surrounded by the prostate. Towards the distal end of this muscular portion is the penis, which has spines but it was not possible to study the number and shape of the spines (Fig. 10e,f). The bursa copulatrix is large and elongate and it could be considered as having two portions. The seminal receptacle is much smaller and narrower than the bursa copulatrix. The seminal receptacle joins the proximal part of vagina via a thin, relatively long, convoluted duct. The wide vagina emerges from the base of the bursa and joins the vas deferens near the genital aperture. A vaginal gland is not present.
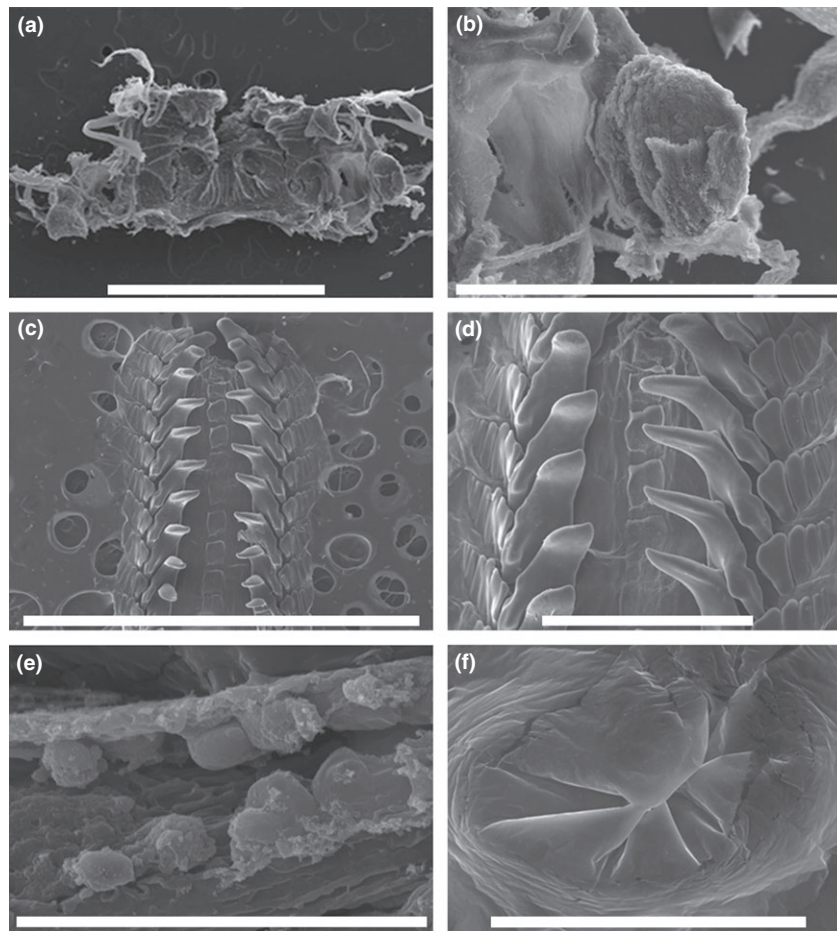
Fig. 10. Scanning electron micrographs of *Tambja divae* (MZSP 103230). (a) Elongate pouches. Scale bar: 1 mm. (b) Detail of the right pouch. Scale bar: 500 μm. (c) Radula. Scale bar: 2 mm. (d) Half radula. Scale bar: 500 μm. (e, f) Penial spines. (e) Scale bar: 50 μm. (f) Scale bar: 100 μm.

Remarks. Tambja divae was described based on a single 12-mm preserved specimen, collected in Arraial do Cabo, south-eastern Brazil (1958). This specimen was recently located in the Marcus collection, at the MZSP (MZSP 76055, Fig. 9b). Since the original description, additional specimens of *T. divae* have not been found and the species has remained poorly known. During a recent expedition to the coast of São Paulo state, a new specimen was collected and it is herein described and compared with the holotype and the original description. The specimen is small but mature, with similar coloration and external features as described by Marcus (1958). The caudal crest described by Marcus is not easy to see, since is not elongate or elevated as in *T. stegosauriformis* (Pola et al., 2005c), but it is present. The gills and rhinophores are also identical in the two specimens. Internally, the tooth morphology of our specimen matches the description and drawings provided by Marcus (1958). Finally, Marcus (1958) gave a complete and detailed description of the reproductive system. Although the interpretation of their words and drawing may be slightly confused, when looking at the reproductive system of the new specimen, Marcus's description is perfectly understandable. In both specimens the vaginal gland is absent and the prostate is morphologically differentiated. In this paper we present the first photograph of a live animal and complete the description with scanning electron micrographs of the radula and the penial spines. We also present a new drawing of the reproductive system and describe the elongate pouches at the junction of the oral tube and the buccal bulb also present in other species of the genus *Tambja*. Curiously, the other species of the genus with elongate pouches are those with the same kind of reproductive system, i.e. a very well-differentiated morphological prostate and without vaginal gland. These species are *T. limaciformis* (Eliot, 1908) and *T. amakusana* Baba, 1987. In our molecular analysis these three species form a separate and very well-supported clade.

Tambja marbellensis Schick and Cervera, 1998 (Figs 9d and 11)

Material examined. Outão, 30 km from Setubal, Setubal, Atlantic Ocean, Portugal, 20 April 2009, 1 specimen, 45 mm preserved, collected by Fatima Mar, 12 m depth on bryozoans (CASIZ 180379).

Distribution. Southern Spain (Schick and Cervera, 1998; Schick, 1998; Ocaña et al., 2000, 2004; Sánchez-Tocino et al., 2000a,b; García-Gómez, 2002) and Portugal (Malaquias and Morenito, 2000; Calado and Silva, 2012; present study).

Remarks. Schick and Cervera (1998) described *Tambja marbellensis* based on two specimens, 12 and 45 mm in length. Their description is quite complete but the authors did not include a picture of the living animal. Although they mention the penial spines, the description lacks scanning electron micrographs of them. Sánchez-Tocino et al. (2000a) found the first nine specimens following the original description, all of them between 3 and 25 mm in length. These authors described only the external coloration and the radular morphology of the specimens, without further details on the reproductive system. Pola et al. (2006b) re-examined the holotype and added further details to expand the original description, such as details of the lateral slots on both sides of the body between the rhinophores and the oral tentacles, also present in other species of the genus, and included scanning electron micrographs of the penial spines. Here an additional 45-mm-long specimen is studied and illustrated (Fig. 9d). We include new micrographs of the smooth labial cuticle, the radula (20 × 4.1.1.1.4) and the penis with numerous penial spines (Fig. 11), confirming the previous known data for this species. In addition we found that internally *T. marbellensis* has the anterior part of the foot full of small, rounded oral glands and externally some spicules can be found scattered over the mantle. It is also the first time that molecular data are available for this species. In our analysis, *Tambja marbellensis* forms a very well-
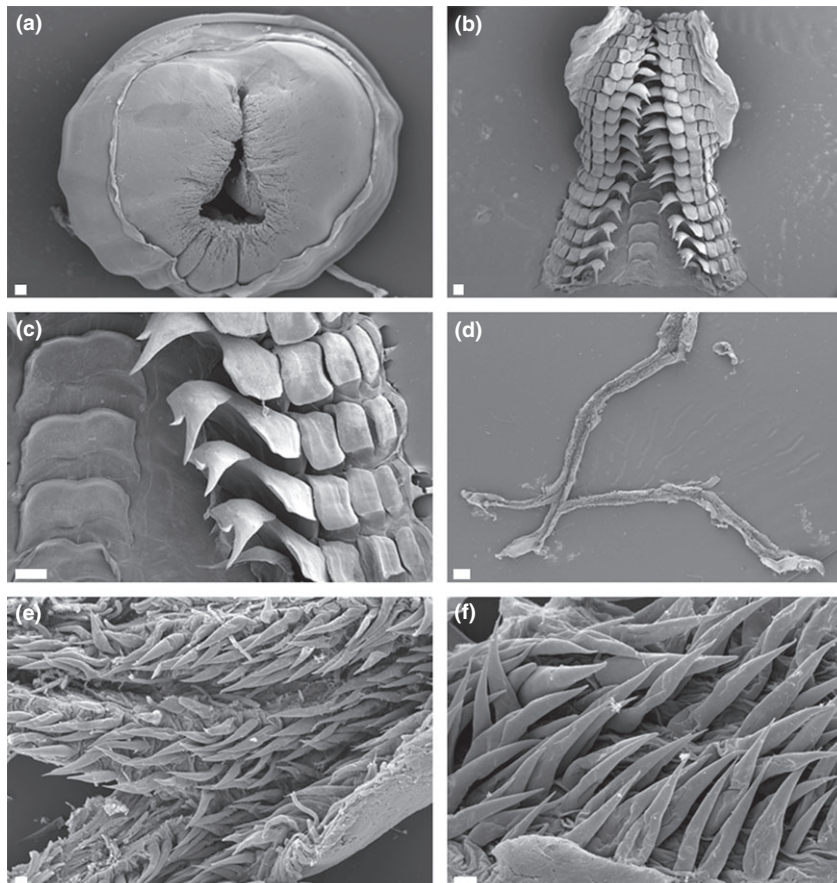

Fig. 11. Scanning electron micrographs of *Tambja marbellensis* (MZSP 180379). (a) Labial cuticle. Scale bar: 100 μm. (b) Radula. Scale bar: 100 μm. (c) Right half of the radula. Scale bar: 100 μm. (d) Penis. Scale bar: 200 μm. (e, f) Penial spines. (e) Scale bar: 10 μm. (f) Scale bar: 10 μm.

supported clade that includes *T. fantasmalis*, *T. crioula* and *T. simplex*, with *T. marbellensis* the sister species to the rest.

Molecular results

In this study, we used a previous molecular phylogeny including 48 specimens (Pola et al., 2008b) and added 21 more specimens, representing 11 additional species of Nembrothinae absent from the previous analysis (Pola et al., 2007, 2008b). For all these specimens we provide *COI*, *16S* and *H3* DNA sequences (Table 1). All new sequences are deposited in GenBank (Table 1).

The combined *COI+16S* dataset yields a sequence alignment of 1075 positions. The *H3+COI+16S* dataset yields a sequence alignment of 1403 positions. The ILD test shows no significant conflicting signal between the two genes ($P = 0.11$). No saturation is observed across genes and codon positions (data not shown). The combined trees provide better resolution than COI, 16S and H3 separately (data not shown).

Figure 12 illustrates the Bayesian consensus tree of the *COI+16S* genes concatenated and includes posterior probabilities (PP) and bootstraps support values from the ML and the MP analysis. Analysis of the combined molecular data set resulted in a tree where Nembrothinae formed a monophyletic group (PP = 1.00, ML = 77, MP = 67) (Fig. 12). Within

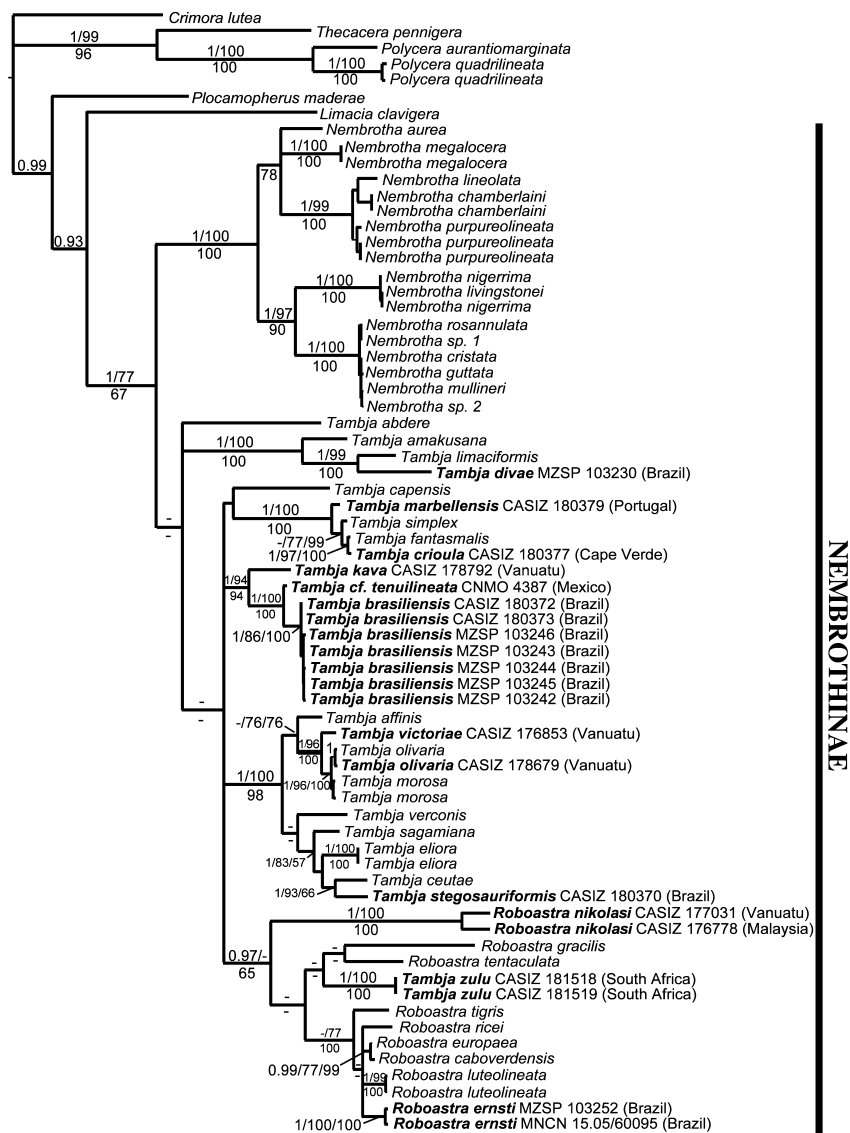Fig. 12. Phylogenetic hypothesis based on combined molecular data (COI+16S) represented by Bayesian inference. Numbers above branches represent, first, posterior probabilities from Bayesian inference and, second, bootstrap values for ML. When numbers appear under branches, they indicate bootstrap values for MP. Specimens in bold refer to the additional 11 species and 21 specimens included in this study.

Nembrothinae, only the genus *Nembrotha* was monophyletic with maximum support (PP = 1.00, ML = 100, MP = 100). Regarding *Roboastra*, its monophyly was broken by the species *Tambja zulu*. This clade [*Roboastra* + *T. zulu*] was supported in Bayesian analysis (PP = 0.97) but not recovered by ML or MP. Within this clade, only two species, *R. europaea* and *R. caboverdensis*, are sister species (PP = 0.97, ML = 77, MP = 99). The remaining species form a polytomy within the clade [*Roboastra* + *T. zulu*].

Regarding *Tambja*, its monophyly was not supported in any of our analyses (Fig. 12). These species formed six highly supported clades within Nembrothinae, more

clearly recognized in Fig. 13. *Tambja abdere* and *T. capensis* did not cluster with any other species. A clade including *T. amakusana*, *T. limaciformis* and *T. divae* was very well supported (PP = 1.00, ML = 100, MP = 100), with *T. limaciformis* and *T. divae* as sister species (PP = 1.00, ML = 100, MP = 100). The fourth clade with maximum support comprised *T. marbellensis*, *T. simplex*, *T. fantasmalis* and the new species described from the Cape Verde Islands with *T. fantasmalis* and *T. crioula* as sister species (PP = 1.00, ML = 97, MP = 100). The next clade was the one including *T. kava*, *T.* cf. *tenuilineata* from Mexico and the new species from Brazil, *T. brasiliensis*. In this case, *T.* cf. *tenuilineata* and *T. brasiliensis* were

Fig. 13. Phylogenetic hypothesis based on combined molecular data (COI+16S) represented by Bayesian inference collapsing branches without support. Numbers above branches represent, first, posterior probabilities from Bayesian inference and, second, bootstrap values for ML. When numbers appear under branches, they indicate bootstrap values for MP. Specimens in bold refer to the additional 11 species and 21 specimens included in this study.

sister species (PP = 1.00, ML = 100, MP = 100) and those two were sister to *T. kava* from Vanuatu (PP = 1.00, ML = 94, MP = 94). Finally, a larger clade strongly supported in all analyses (PP = 1.00, ML = 100, MP = 98) included the remaining studied species of *Tambja*. Within this clade *T. affinis* and *T. verconis* did not cluster with any other species with a high degree of support. The other species were included in two separate subclades, one including *T. victoriae*, *T. olivaria* and *T. morosa* (PP = 1.00, ML = 96, MP = 100), and the other including *T. sagamiana*, *T. eliora*, *T. ceutae* and *T. stegosauriformis* (PP = 1.00, ML = 83, MP = 57).

For comparison purposes, we also ran the same analyses including H3 sequences, but only 21 sequences were available for H3 as Pola et al. (2008b) did not include this gene. In this case, the Bayesian consensus tree of the dataset *H3+COI+16S* (Fig. 14) differed in two major points. First, within Nembrothinae, in addition to the maximum supported *Nembrotha* clade, there was another well-supported clade containing [*Roboastra* + *T. zulu*] and all *Tambja* species except for *T. abdere*, and the clade including *T. amakusana*, *T. limaciformis* and *T. divae*. This clade was supported in Bayesian analysis (PP = 0.99) but not recovered by ML or MP. The second major differ-
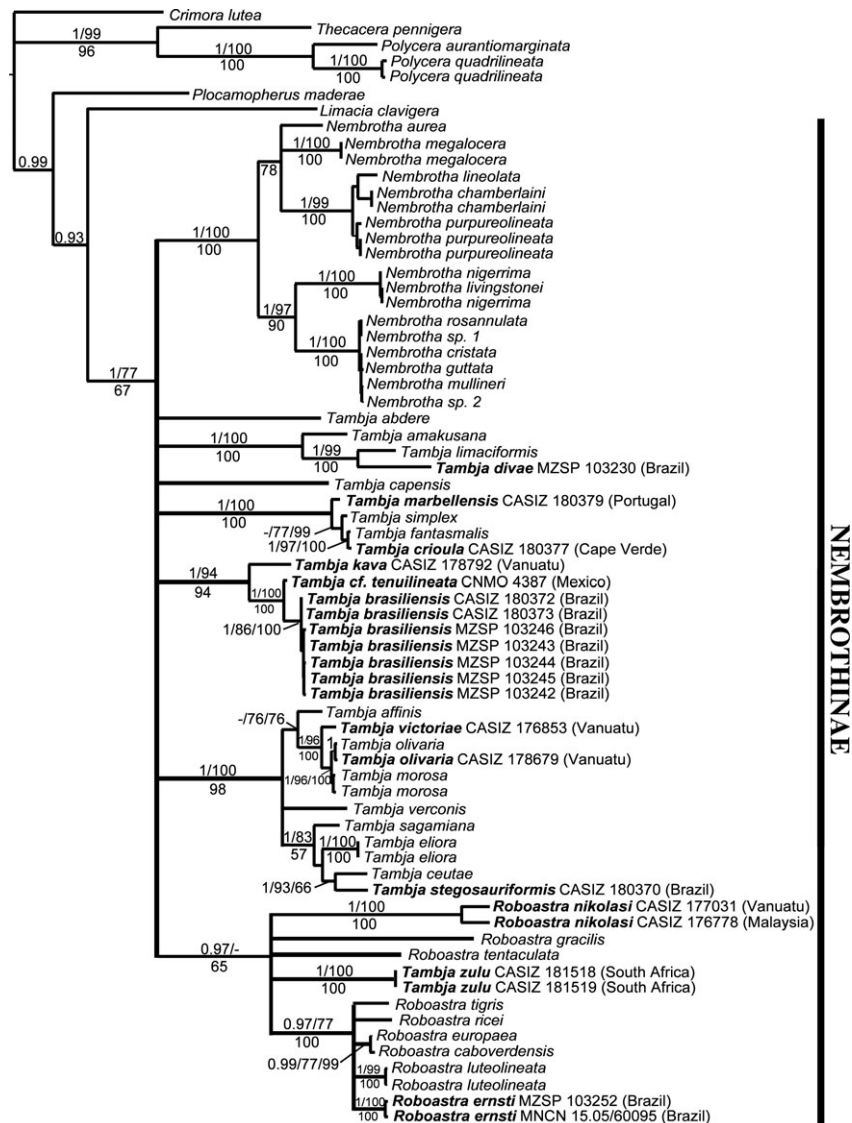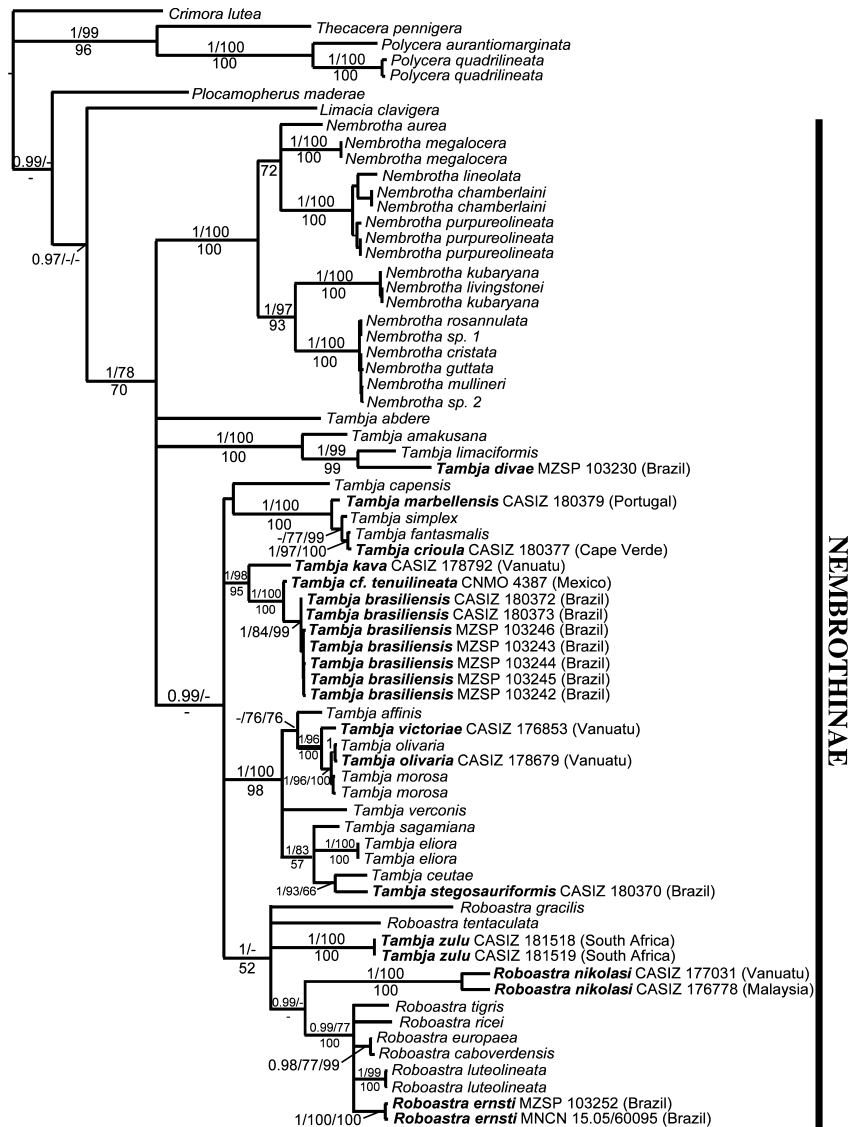

Fig. 14. Phylogenetic hypothesis based on combined nuclear and molecular data (H3+COI+16S) represented by Bayesian inference collapsing branches without support. Numbers above branches represent, first, posterior probabilities from Bayesian inference and, second, bootstrap values for ML. When numbers appear under branches, they indicate bootstrap values for MP. Specimens in bold refer to the additional 11 species and 21 specimens included in this study.

ence was that, in this case, within the well-supported clade [*Roboastra* + *T. zulu*], *R. tigris*, *R. luteolineata*, *R. ernsti*, *R. ricei*, *R. europaea* and *R. caboverdensis* formed a highly supported clade (PP = 0.99, ML = 77, MP = 100) and all these species are sister to *R. nikolasi*. This latter relationship was supported only in Bayesian analysis (PP = 0.99, ML = −, MP = −).

Discussion

This study includes 21 more specimens, representing 11 additional species of Nembrothinae absent from the previous analysis (Pola et al., 2007, 2008b), in an attempt to clarify the phylogenetic relationships within the subfamily. However, we have again failed to fully resolve the phylogeny of the Nembrothinae. Clearly, Nembrothinae is monophyletic, but the systematics of the genera included within it to date is far from being completely understood. So far, within Nembrothinae, the genus *Nembrotha* is the only one clearly recovered as monophyletic. Members of this genus are found only in the Indo-Pacific and seem to have diverged from the other nembrothines early in their evolution. In addition, all *Nembrotha* species feed on tunicates while the remaining species of Nembrothinae have different preferences for food. No new species of *Nembrotha* are included in this study so we do not discuss this clade in more detail.

One of the major problems related to this group is the large number of species yet to be found and studied. Evidence of the existence of many intermediate colour forms and undescribed species can be found in different guide books or webpages as *Tambja* sp.2, sp.6, sp. 8, sp. 9, sp. 10, sp. 11; *Nembrotha* sp.1, sp.2, sp.5, sp.8, sp. 11, sp. 13, sp.15 (www.seaslugforum.net; www.nudipixel.net, among many others) and, looking at our last phylogeny presented herein, they are completely necessary for understanding the evolutionary relationships within this group. Intermediate missing species seem to be critical to help us to explain why a species such as *Tambja zulu* fits within the genus *Roboastra*. Pola et al. (2008b) transferred *Tambja tentaculata* to *Roboastra* based on the presence of very well-developed grooved laterally oral tentacles, leaving this character as the only synapomorphy supporting the genus *Roboastra*. In this case, we have observed in the recently collected and studied specimens of *T. zulu* (MNCN 15.05/68505) that those very well-developed oral tentacles are also present (Fig. 9e) and thus the most parsimonious solution would be to transfer *T. zulu* to *Roboastra zulu*, following the decision taken by Pola et al. (2008b). Thus, *Roboastra* would remain as a clade (Fig. 13). However, we are no longer sure if this is the best hypothesis. The well-known species of *Roboastra*, among them *R. europaea*, *R. caboverdensis*,

R. tigris, *R. luteolineata*, *R. leonis* and *R. ricei*, are known to prey upon species of *Tambja*. In addition, the new species described from Brazil also feeds on species of *Tambja*; in Fig. 1d it is shown feeding on *T. stegosauriformis*. All these species are large and robust and have what has traditionally been considered the typical radula of *Roboastra*, with very well-developed rachidian and lateral teeth, both having large, sharp denticles. However, to date, there are no feeding observations from species such as *R. tentaculata*, *R. nikolasi* or *T. zulu*. There is a record of *R. gracilis* eating *Nembrotha kubaryana* (Gudgeon, 2006) but looking closely at the pictures we are not sure that it is not a juvenile of *R. luteolineata*. In fact, in most of the photographs of *R. gracilis* shown in the seaslugforum, this species appears crawling above what seems to be bryozoans (Osmond, 2000; Chan, 2002; Krampf, 2007; Dixon, 2007) (Fig. 9f,g). None of the photographers who documented this species mentioned the food, but clearly they are not feeding on any species of *Tambja*. In this study we present the first observations of *Roboastra tentaculata* feeding on bryozoan colonies (Fig. 9h). In fact, in Fig. 9e *Tambja zulu* seems to be feeding on bryozoans too but this appreciation is not as clear. These first records confirm that in-depth knowledge of the feeding behaviour of the not as robustly defined *Roboastra* is necessary to understand the relationships among all these species. In addition, the lateral slots present in some *Tambja* species (and also present in *R. tentaculata*) are visible in these specimens of *T. zulu* (Fig. 9e). The large and robust species of *Roboastra* previously mentioned do not have these lateral slots. In addition, within the clade [*Roboastra* + *T. zulu*] the difference found between both datasets, excluding or including H3 sequences, may be an artefact of the missing data. The well-supported clade including *R. tigris*, *R. luteolineata*, *R. ernsti*, *R. ricei*, *R. europaea* and *R. caboverdensis* should be tested when including all H3 sequences. Exactly the same is true for the clade including [*Roboastra* + *T. zulu*] and all *Tambja* species except *T. abdere*, and the clade including *T. amakusana*, *T. limaciformis* and *T. divae*.

Our analyses show that, to date, the genus *Tambja* is not monophyletic, as already shown by Pola et al. (2007, 2008b). We know that there are many additional species awaiting discovery and description. In addition, new characters, such as the presence of spicules in the mantle, may be significant and should be carefully studied. Pola et al. (2007) underestimated this character as it seems that spicules disappear when the specimens are preserved in formalin and thus this character was not observed in many of the specimens by the authors. Fresh material frozen or directly preserved in alcohol allows us to clearly detect the spicules, as occurs in the present study. We suggest

some new information to be taken in account. First, *T. verconis* is the type species of the genus, and *T. stegosauriformis* and *T. victoriae* (new additions in this study) are members of the genus *Tambja sensu stricto* because they cluster together in the same clade and with maximum support. Next, the recently redis-covered Atlantic *Tambja divae* is closely related to *T. amakusana* and *T. limaciformis* from the Indo-Paci-fic. This is a significant finding as these three species are the only members of the genus *Tambja* sharing elongate pouches at the junction of the oral tube and the buccal bulb and have the same kind of reproduc-tive system, having a very well-differentiated morpho-logical prostate and without a vaginal gland. Moreover, this is the first time that DNA sequences of *Tambja marbellensis* and *T. crioula* have been avail-able. *Tambja marbellensis* is the sister species to a clade containing *T. simplex*, *T. fantasmalis* and *T. crioula*. As discussed under the remarks for *T. crioula*, all these species are very similar internally but coloration allows us to distinguish them. One possible explanation is that all these species share the same distribution and suggests that sufficient time has not elapsed for mor-phological divergence, as the genetic distances among them are very low (Ornelas-Gatdula and Valdés, 2012). Finally, a strongly supported new clade is formed by two new species described in this paper (*T. kava* and *T. brasiliensis*) and a third species identi-fied as *T.* cf. *tenuilineata*, from Mexico.

In summary, as there are many additional species still to be described and studied and they may be criti-cal to understand the evolutionary relationships within this group, we can only interpret our own results as a work in progress. The Nembrothinae is revealed to be an intricate and challenging group of nudibranchs to study. Despite their beautiful coloration and large size, phylogenetic relationships and systematic relationships remain elusive. Slowly but surely adding additional taxa and additional characters will allow us to further explore a more complete taxonomic and phylogenetic revision of the family. Based on the present results, we have decided not to change the traditional classifica-tion so as to not create even more confusion in the short term. Further studies with more complete taxon sampling need to be undertaken prior to making addi-tional taxonomic decisions.

Acknowledgements

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3. RESULTS

<u>Chapter 9</u>

Goodheart JA, Camacho-García Y, **Padula V**, Schrödl M, Cervera JL, Gosliner TM, Valdés Á (2015) Systematics and biogeography of *Pleurobranchus* Cuvier, 1804 sea slugs (Mollusca: Heterobranchia: Nudipleura: Pleurobranchidae). *Zoological Journal of the Linnean Society*. DOI: 10.1111/zoj.12237.

Systematics and biogeography of *Pleurobranchus* Cuvier, 1804, sea slugs (Heterobranchia: Nudipleura: Pleurobranchidae)

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Species of *Pleurobranchus* (Mollusca: Gastropoda: Heterobranchia: Nudipleura: Pleurobranchidae) are commonly found worldwide, but there is a substantial amount of confusion regarding the ranges and identification of individual species. Difficulties in phylogenetic reconstruction and identification of pleurobranchids using morphological traits has resulted in complex classification schemes, with several species having disjunct ranges across physical and biogeographical barriers (including the tropical Indo-Pacific, the eastern Pacific, and the Atlantic). A sizeable number of species of *Pleurobranchus* has been described; however, many of these species are morphologically and biogeographically similar to others, and probably constitute synonyms. This paper provides a phylogenetic framework of classification for *Pleurobranchus* based on the mitochondrial genes *cytochrome c oxidase I* (*COI*) and *16S* rDNA and the nuclear gene *histone 3* (*H3*) using Bayesian and maximum likelihood approaches. Molecular phylogenies obtained recovered most of the well-established species of *Pleurobranchus* and some morphological characters were found to have taxonomic value for delimiting species in this group. Automatic barcode gap discovery (ABGD) analyses substantiated the distinctiveness of units/species recovered in the phylogenetic analyses, with some exceptions. Morphological descriptions for the 14 species recovered in the molecular phylogeny and discussions on the biogeography and colour variation are included.

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INTRODUCTION

Pleurobranchus (Nudipleura, Pleurobranchidae) is a group of side-gilled heterobranch sea slugs, typically found intertidally and in the shallow subtidal of tropical and subtropical regions worldwide. According to Willan (1987), the main synapomorphies of *Pleurobranchus* are the presence of a tuberculate mantle and gill rachis, a cleft anterior border of the mantle, and flaps surrounding the genital aperture in mature adults. The monophyly of *Pleurobranchus* has been confirmed by morphoanatomical cladistics approaches (Willan, 1987; Cervera *et al*., 2000; Martynov & Schrödl, 2009). Martynov & Schrödl (2009) provided evidence that *Pleurobranchus* is a derived rather than a basal pleurobranchoidean clade, which was subsequently confirmed by Göbbeler & Klussmann-Kolb (2010). Species of *Pleurobranchus* are exclusively carnivorous and have a reduced internal shell. Some species of *Pleurobranchus* secrete chemicals for defence that are potentially beneficial to human health, including *Pleurobranchus albiguttatus* and *Pleurobranchus forskalii*, which contain compounds (cytotoxic lissoclimide-type diterpenes) that have shown promising toxicity against melanomas and solid tumours (Fu *et al*., 2004).

Over 70 species of *Pleurobranchus* (including synonyms) have been identified and described, many based solely on characteristics of the internal shell and some on external coloration (Martynov & Schrödl, 2009). As the shell of *Pleurobranchus* lacks distinctive traits (Willan, 1987), the recognition and identification of many of these species is problematic. In recent years, experts have disagreed on the value of certain morphological traits for classification and phylogenetic reconstruction of pleurobranchids (Willan, 1987; Gosliner & Bertsch, 1988), which also has implications for our ability to recognize and identify species.

Difficulties in phylogenetic reconstruction and identification of species in *Pleurobranchus* using morphological traits has resulted in complex classification schemes, with several species having disjunct ranges across physical and biogeographical barriers. A good example of a problematic taxon is the species *Pleurobranchus areolatus*, which has been reported in both the Caribbean Sea and eastern Pacific, despite the fact that these two regions have been separated for more than 3.1 million years by the formation of the Isthmus of Panama (Coates & Obando, 1996). By contrast, some species seem to have more restricted ranges and are found only in specific localities, such as *Pleurobranchus reticulatus*, which is reportedly found only in the Gulf of Guinea, off the coast of western Africa (Rang, 1832; Neves, Cervera & Calado, 2007), and *Pleurobranchus membranaceus*, which is found exclusively in the Mediterranean and north-eastern Atlantic (Bergh, 1897; Cervera *et al*., 2006).

Of the over 70 published species names of *Pleurobranchus*, only 14 have been used routinely in field guides and recent publications. These include *P. membranaceus* (Montagu, 1815) and *Pleurobranchus testudinarius* Cantraine, 1835, from the Mediterranean, *Pleurobranchus garciagomezi* Cervera, Cattaneo-Vinetti & Edmunds, 1996, and *P. reticulatus* Rang, 1832, from the eastern Atlantic, *Pleurobranchus evelinae* Thompson, 1977, from the Caribbean, *Pleurobranchus areolatus* Mörch, 1863, from the eastern Pacific, Caribbean, and eastern Atlantic, *Pleurobranchus hilli* (Hedley, 1894) from Southern Australia, and *P. albiguttatus* (Bergh, 1905), *P. forskalii* Rüppell & Leuckart, 1828, *Pleurobranchus grandis* Pease, 1868, *Pleurobranchus mamillatus* Quoy & Gaimard, 1832, *Pleurobranchus nigropunctatus* (Bergh, 1907), *Pleurobranchus peronii* Cuvier, 1804, and *Pleurobranchus weberi* (Bergh, 1905) from the Indo-Pacific. Of other species assigned to *Pleurobranchus*, some have been transferred to either *Berthella* or *Berthellina* by previous publications (Burn, 1962; Macnae, 1962; MacFarland, 1966; Edmunds & Thompson, 1972; Thompson, 1977; Ev. Marcus, 1984; Willan, 1984; Willan & Bertsch, 1987; Gosliner & Bertsch, 1988).

The main focus of this paper is to reconstruct the phylogeny of *Pleurobranchus* based on molecular data. We have attempted to clarify the taxonomic status of the existing species within *Pleurobranchus*, including the *P. areolatus* species complex, using two mitochondrial genes [*16S* rDNA and *cytochrome c oxidase I* (*COI*)] and one nuclear gene (*histone 3*, *H3*). Additionally, some morphological traits were reexamined for some species and combined with the molecular data. This paper also includes a review of the relevant literature to determine valid names for each species in *Pleurobranchus* in order to achieve nomenclatural stability for this group.

MATERIAL AND METHODS

Specimens

DNA from 167 specimens of *Pleurobranchus* was extracted, but sequences were obtained for only 72 (Table 1). Forty-five specimens were obtained from the collections of the Natural History Museum of Los Angeles County (LACM), 68 specimens from the California Academy of Sciences Invertebrate Zoology collection (CASIZ) in San Francisco, one specimen from the Natural History Museum of Crete (NHMC), seven specimens from Zoologische Staatssammlung, Munich (ZSM), five specimens from the Museo de Zoología, University of Costa Rica (MZUCR), two specimens from the Zoological Museum, University of Bergen (ZMBN), and two specimens from the Museu de Zoologia, Universidade de São Paulo (MZSP). Twenty-two

Table 1. List of specimens examined in this paper for which sequences were obtained, including locality, museum collection numbers, and GenBank accession numbers for the three genes sequenced. The first column indicates the preliminary identification of the specimen based on available literature and the external colour of the animals. The last column indicates the final identification based on molecular and anatomical data. Sequences shorter than 200 bp were not deposited in GenBank but are marked with an asterisk and provided in Appendix S2

Preliminary identification	Locality	Voucher no.	GenBank accession no.			Final identification
			COI	H3	16S	
Pleurobranchaea meckelii	–	GenBank	FJ917499	EF133470	FJ917439	–
Pleurehdera haraldi	Sand Island, Palmyra Atoll	CASIZ 174202	KM521726	KM521620	–	–
Berthella stellata	St James, Jamaica	CPIC 00655	KM521691	KM521621	KM521594	–
Berthella stellata	Islan Canal de Afuera, Panama	LACM 153343	KM521692	KM521622	–	–
Berthella sp.	Muros de Nalón, Asturias, Spain	CPIC 00445	KM521693	KM521623	–	–
Berthellina delicata	Maui, Hawaii, USA	CPIC 00349	KM521689	KM521624	KM521593	–
Pleurobranchus albiguttatus	Honokeana Bay, Maui, Hawaii, USA	CPIC 00352	–	KM521688	–	*Pleurobranchus varians*
P. albiguttatus	Maliko Bay, Maui, Hawaii, USA	CPIC 00357	KM521701	KM521626	KM521598	*P. varians*
P. albiguttatus	Honokeana Bay, Maui, Hawaii, USA	CPIC 00409	KM521702	KM521627	KM521599	*P. varians*
P. albiguttatus	Madang, Papua New Guinea	CASIZ 191409	KM521736	KM521657	–	*P. albiguttatus*
P. albiguttatus	Honokeana Bay, Maui, Hawaii, USA	CPIC 00351	KM521700	KM521625	KM521597	*P. varians*
P. albiguttatus	Plettenberg Bay, South Africa	CASIZ 075983	–	KM521682	KM521612	*Pleurobranchus nigropunctatus*
P. albiguttatus	Madang, Papua New Guinea	CASIZ 191076	KM521746	KM521662	–	*P. albiguttatus*
Pleurobranchus areolatus	Yucatán Peninsula, México	CPIC 00196	KM521711	KM521629	–	*P. areolatus*
P. areolatus	Yucatán Peninsula, México	CPIC 00208	KM521712	KM521630	KM521604	*P. areolatus*
P. areolatus	Bahamas	LACM 173235	KM521706	KM521628	–	*P. areolatus*
P. areolatus	La Paz, Baja California, México	LACM A.9555	KM521735	KM521644	–	*Pleurobranchus digueti*
P. areolatus	Jalisco, México	LACM A.8477	–	KM521683	*	*P. digueti*
P. areolatus	Costa Rica, Pacific, Cocos Island	CASIZ 073374	KM521718	KM521645	–	*P. digueti*
P. areolatus	Costa Rica	MZUCR 6178	KM521720	KM521643	–	*P. digueti*
P. areolatus	Costa Rica	MZUCR 6990	KM521721	KM521642	–	*P. digueti*
P. areolatus	Panama	MZUCR 6201	KM521724	KM521647	–	*P. digueti*
P. areolatus	Jalisco, México	CASIZ 175782	KM521725	KM521656	–	*P. digueti*
P. areolatus	Guanacaste, Costa Rica	CASIZ 175736	KM521719	KM521640	–	*P. digueti*
P. areolatus	Costa Rica	MZUCR 6994	KM521722	KM521641	–	*P. digueti*
P. areolatus	Panama	MZUCR 6986	KM521723	KM521646	–	*P. digueti*
P. areolatus	Espiritu Santo Island, Vanuatu	CASIZ 175771	KM521705	KM521650	–	*P. varians*
P. areolatus	Costa Rica	–	KM521715	KM521639	–	*P. areolatus*
P. areolatus	Costa Rica	–	KM521714	KM521638	–	*P. areolatus*
P. areolatus	Panama	CPIC 00929	KM521709	KM521649	–	*P. areolatus*
P. areolatus	Panama	–	KM521710	KM521648	–	*P. areolatus*
P. areolatus	Brazil	MZSP103373	KM521737	KM521659	–	*Pleurobranchus reticulatus*
P. areolatus	Brazil	MZSP103380	KM521738	KM521660	–	*P. reticulatus*
Pleurobranchus crossei	Bahamas	LACM 173236	KM521708	KM521631	KM521605	*P. areolatus*
P. crossei	Florida, USA	CPIC 00829	KM521707	KM521681	–	*P. areolatus*
P. crossei	Palm Beach, Florida, USA	CPIC 00847	KM521753	–	*	*P. areolatus*
Pleurobranchus evelinae	Conch Point, Isla de Colon, Panama	LACM 2004–12.1	KM521713	KM521632	KM521606	*P. areolatus*
Pleurobranchus forskalii	Great Barrier Reef, Australia	CASIZ 078704	KM521727	KM521663	KM521603	*P. forskalii*

Table 1. *Continued*

Preliminary identification	Locality	Voucher no.	COI	H3	16S	Final identification
P. forskalii	Madang, Papua New Guinea	CASIZ 191034	KM521739	KM521668	–	*P. forskalii*
P. forskalii	Madang, Papua New Guinea	CASIZ 191164	KM521740	KM521669	–	*P. forskalii*
P. forskalii	Philippines	–	KM521729	KM521666	–	*P. forskalii*
P. forskalii	Philippines	CASIZ 176077	KM521728	KM521667	–	*P. forskalii*
Pleurobranchus garciagomezi	Boca das Caldirinhes, Faial Islands, Azores	ZMBN 81687	–	KM521686	–	*P. reticulatus*
P. garciagomezi	Faial Island, Azores	ZMBN 81685	–	KM521685	–	*P. reticulatus*
P. garciagomezi	São Vicente, Cape Verde	–	KM521754	KM521613	–	*P. reticulatus*
P. garciagomezi	Boavista, Cape Verde	–	–	KM521614	–	*P. reticulatus*
P. garciagomezi	Canary Islands, Spain	–	KM521744	–	–	*P. reticulatus*
Pleurobranchus grandis	Luzon Islands, Philippines	CASIZ 177651	KM521698	KM521633	KM521596	*P. ueberi*
P. grandis	New Caledonia	–	KM521697	KM521652	–	*P. grandis*
P. grandis	Sulawesi, Indonesia	ZSM 20034031	KM521696	KM521678	KM521609	*P. grandis*
Pleurobranchus hilli	Australia	GenBank	FJ917497	–	FJ917438	*P. hilli*
Pleurobranchus mamillatus	Maalaea Bay, Maui, Hawaii, USA	CPIC 00818	KM521747	KM521661	–	*P. mamillatus*
Pleurobranchus membranaceus	Ardrichaig Pinnacles, Loche Fyne, Scotland	–	KM521748	KM521675	–	*P. membranaceus*
P. membranaceus	Fraoch Eilean, Minard Island, Loche Fyne, Scotland	–	KM521749	KM521676	–	*P. membranaceus*
P. membranaceus	Fraoch Eilean, Minard Island, Loche Fyne, Scotland	–	KM521750	KM521677	–	*P. membranaceus*
P. membranaceus	France	GenBank	FJ917496	–	FJ917437	*P. membranaceus*
P. membranaceus	France	ZSM 20070301	KM521752	KM521680	KM521611	*P. membranaceus*
P. membranaceus	Croatia	ZSM 20100634	KM521751	KM521679	KM521610	*P. membranaceus*
Pleurobranchus peronii	Maliko Bay, Maui, Hawaii, USA	CPIC 00358	KM521703	KM521664	KM521600	*P. varians*
P. peronii	Kapalua Bay, Maui, Hawaii, USA	CPIC 00405	KM521704	KM521665	KM521601	*P. varians*
P. peronii	Luzon Islands, Philippines	CASIZ 177491	KM521716	KM521672	KM521607	*P. peronii*
P. peronii	Luzon Islands, Philippines	CASIZ 177440	KM521730	KM521673	KM521608	*P. peronii*
P. peronii	Madang, Papua New Guinea	CASIZ 191141	KM521741	KM521658	–	*P. albiguttatus*
P. peronii	Madang, Papua New Guinea	CASIZ 191300	KM521742	KM521670	–	*P. forskalii*
P. peronii	Madang, Papua New Guinea	CASIZ 191359	KM521743	KM521671	–	*P. forskalii*
P. peronii	Madang, Papua New Guinea	CASIZ 191434	KM521745	KM521674	–	*P. peronii*
P. reticulatus	Guinea	GenBank	FJ917498	–	–	*P. reticulatus*
P. ueberi	Luzon Islands, Philippines	CASIZ 181282	KM521699	KM521651	–	*P. ueberi*
Pleurobranchus sp.	Honokeana Bay, Maui, Hawaii, USA	CPIC 00342	KM521717	KM521636	KM521602	*P. albiguttatus*
Pleurobranchus sp.	Kapalua Bay, Maui, Hawaii, USA	CPIC 00404	KM521731	KM521637	KM521595	*P. peronii*
Pleurobranchus sp.	Indonesia	CASIZ 139587	KM521732	KM521654	–	*P. peronii*
Pleurobranchus sp.	Hawaii, USA	CASIZ 166753	KM521733	KM521655	–	*P. peronii*
Pleurobranchus sp.	Australia	–	KM521734	KM521653	–	*P. peronii*
Pleurobranchus sp.	Greece	CPIC 00444	KM521694	KM521634	–	*Berthellina* sp.
Pleurobranchus sp.	Costa Rica	–	KM521690	KM521635	–	*Berthellina* sp.
Pleurobranchus sp.	Crete, Greece	NHMC 52.37	–	KM521687	–	*P. testudinarius*
Pleurobranchus sp.	Cagarras, Brazil	–	–	KM521619	–	*P. testudinarius*
Pleurobranchus sp.	Gales, Brazil	–	–	KM521618	–	*P. testudinarius*

COI, cytochrome c oxidase I; H3, histone 3.

specimens were obtained by direct collection via snorkelling and SCUBA diving and were deposited in the Cal Poly Pomona Invertebrate Collection (CPIC). All specimens deposited at these museums were collected and exported with appropriate permits from each country in which collections were made. Fifteen specimens do not have voucher numbers from any institution, as only small tissue samples were taken from these animals before they were returned to the ocean. All specimens were preserved in 70 or 95% ethanol for long-term storage. Sequences for four individuals were downloaded from GenBank, *Pleurobranchaea meckelii* (FJ917499, EF133470, FJ917439), *P. hilli* (FJ917497, FJ917438), *P. membranaceus* (FJ917496, FJ917437), and *P. reticulatus* (FJ917498).

DNA EXTRACTION

DNA extraction was performed using either a hot Chelex protocol or DNeasy Blood and Tissue Kits (Qiagen). Approximately 1–3 mg tissue was taken from the foot of the animals and cut into fine pieces for extraction for each protocol. For the Chelex extraction, the tissue was rinsed and rehydrated using 1.0 mL Tris-EDTA (TE) buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0) for 20 min. A 10% (w/v) Chelex 100 (U.S. Standard 100–200 mesh, sodium form, Bio-Rad) solution was prepared using TE buffer. After rehydration, the mixture was then centrifuged, 975.00 µL of the supernatant was removed, and 175.00 µL of the Chelex solution was added. Samples were then incubated at 56 °C in a water bath for 20 min, heated to 100 °C in a heating block for 8 min, and the supernatant was used for PCR.

The DNeasy protocol supplied by the manufacturer was followed, with some modifications. The elution step was modified such that the first elution was collected using 100.00 µL Buffer AE and was allowed to incubate at room temperature for 5 min. In a new tube, a second elution step was conducted using 200.00 µL Buffer AE and was also allowed to incubate at room temperature for 5 min. The first elution, and in some cases the second elution, was used for PCR. Multiple extraction protocols were implemented owing to the fact that some tissue samples could not be amplified when using a particular extraction method. The use of the DNeasy extraction protocol was only used as an alternative to the Chelex extraction when it became apparent that the DNA could not be successfully extracted using the latter technique.

PRIMERS

Colgan's universal *H3* primers (Colgan *et al.*, 1998), Folmer's universal *COI* primers (Folmer *et al.*, 1994), and Palumbi's universal *16S* primers (Palumbi, 1996) were used to amplify the regions of interest for some specimens (Table 2). However, internal primers for *16S* and external primers (as compared with the universal primers) for *COI* designed for another group of sea slugs (Ornelas-Gatdula, Dupont & Valdés, 2011) were also used, resulting in additional partial sequences for some specimens.

PCR AMPLIFICATION AND SEQUENCING

The master mix (for each sample) was prepared using 34.75 µL H$_2$O, 5.00 µL PCR buffer (ExACTGene, Fisher Scientific), 5.00 µL 25 mM MgCl$_2$, 1.00 µL 40 mM deoxynucleotide triphosphates, 1.00 µL 10 µM primer 1, 1.00 µL primer 2, 0.25 µL 5 mg mL^{-1} Taq, and 2.00 µL extracted DNA. Reaction conditions for *H3* and *16S*

Table 2. Forward (F) and reverse (R) PCR primers used to amplify regions of the nuclear *histone 3* (*H3*) gene and mitochondrial *cytochrome c oxidase* I (*COI*) and *16S* genes

Name	Sequence 5′–3′	Source
H3		
HexAF (F)	ATG GCT CGT ACC AAG CAG ACG GC	Colgan *et al.* (1998)
HexAR (R)	ATA TCC TTG GGC ATG ATG GTG AC	Colgan *et al.* (1998)
16S rRNA		
16Sar-L (F)	CGC CTG TTT ATC AAA AAC AT	Palumbi *et al.* (1996)
16Sbr-H (R)	CCG GTC TGA ACT CAG ATC ACG T	Palumbi *et al.* (1996)
16Sar-FAP (F)	AAA GAC GAG AAG ACC CTT AGA GTT TT	Ornelas-Gatdula *et al.* (2011)
16Sbr-FAP (R)	AAA ACT CTA AGG GTC TTC TCG TCT TT	Ornelas-Gatdula *et al.* (2011)
COI		
LCO1490 (F)	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.* (1994)
HCO2198 (R)	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)
LCO-NAF (F)	GCC TTT TCA ACA AAC CAT AAA GA	
HCO-NAR (R)	CCA TCC TGG TAA AAT TAA AAT ATA	

rRNA were as follows: an initial denaturation for 2 min at 94 °C, 35 cycles of (1) denaturation for 30 s at 94 °C, (2) annealing for 30 s at 50 °C, and (3) elongation for 1 min at 72 °C, and a final elongation for 7 min at 72 °C. Reaction conditions for *COI* were an initial denaturation for 3 min at 95°C, 39 cycles of (1) denaturation for 45 s at 94°C, (2) annealing for 45 s at 45°C, and (3) elongation for 2 min at 72°C, and a final elongation for 10 min at 72°C. PCR products yielding bands of appropriate size (approximately 375 bp for *H3*, 195 bp for the *16S* fragments, and 695 bp for *COI*) were purified using the Montage PCR Cleanup Kit (Millipore).

Cleaned PCR samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Each primer was diluted to 4.0 µM to send out for sequencing with the PCR products. PCR products were diluted to between 5 and 30 ng µL^{-1} for sequencing. Sequencing was outsourced to Eton Biosciences (San Diego, CA).

SEQUENCE ANALYSES

Sequences for each gene were assembled and edited using GENEIOUS PRO 4.8.5 (Drummond *et al*., 2009). GENEIOUS was also used to extract the consensus sequence and to construct the alignment for each gene using the default parameters. The sequences were not trimmed after alignment. The total length of *H3* was 328 bp, approximately 450 bp for *16S*, and 658 bp for *COI*. A combined analysis was conducted using all genes concatenated (Fig. 1).

The levels of saturation for each gene were investigated using the substitution saturation test developed by Xia *et al*. (2003) and Xia & Lemey (2009) implemented in the program DAMBE (Xia & Xie, 2001).

To assess whether *COI*, *H3*, and *16S* have significantly conflicting signals, the incongruence length difference (ILD) test (Mickevich & Farris, 1981; Farris *et al*., 1994), implemented in PAUP*4.0 as the partition homogeneity test (Swofford, 2002), was conducted for all three genes combined.

Bayesian and maximum likelihood analyses were conducted for all genes concatenated and for each gene separately. To determine the best-fit model of evolution for the Bayesian analysis, the Akaike information criterion (Akaike, 1974) was executed in MrModeltest v. 2.3 (Nylander, 2004), using PAUP*4.0 (Swofford, 2002; Table 3). The Bayesian analyses were implemented in MrBayes 3.2.1 (Huelsenbeck & Ronquist, 2001), with partitioning by gene (unlinked). The Metropolis-coupled Markov chain Monte Carlo analysis was run with two runs of six chains for 10 000 000 generations, with sampling every 100 generations, and a default burn-in of 25%. Clades with posterior probabilities ≥ 0.95 ($\alpha = 0.05$) were considered significant (Alfaro, Zoller & Lutzoni, 2003). The

maximum likelihood analyses were implemented in GARLI 2.0 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006) and grid computing (Cummings & Huskamp, 2005) through The Lattice Project (Bazinet & Cummings, 2008), which created a web service for GARLI (Bazinet & Cummings, 2011) that uses a special programming library and associated tools (Bazinet *et al*., 2007). Following the general computational model of a previous phylogenetics study (Cummings *et al*., 2003), which used an earlier grid computing system (Myers & Cummings, 2003), the required files were distributed amongst hundreds of computers where the analyses were conducted asynchronously in parallel. Partitioned maximum likelihood analyses were conducted on the total data matrix, with each partition set to its optimal model (as described above), with these models unlinked and employing their own rates (i.e. using the settings linkmodels = 0 and subsetspecificrates = 1). A bootstrap analysis was run with 2000 replicates under the default parameters of the web service. Postprocessing of the phylogenetic inference results was carried out using DendroPy (Sukumaran & Holder 2010) and the R system for statistical computing (R Development Core Team, 2011). The estimation of the number of replicates required to recover the 'best' topology follows Regier *et al*. (2009). *Pleurobranchaea meckelii* was used as the outgroup for both analyses because of the hypothetical basal position of *Pleurobranchaea* with respect to *Pleurobranchus* (Willan, 1987).

Date estimates for uncalibrated nodes in the Bayesian consensus tree were derived by using r8s 1.7 (Sanderson, 2003) using the finalization of the closure of the Isthmus of Panama as the calibration point, estimated as 3.1–3.5 Mya (Coates & Obando, 1996). Several analyses using the Langley–Fitch, nonparametric rate, and penalized likelihood methods were run. For the Langley–Fitch method, analyses were run using the Powell, truncated Newton, and quasi-Newton algorithms as well as the local molecular clock procedure. The nonparametric rate method analysis was run with the Powell algorithm and the penalized likelihood analyses with the Powell and truncated Newton algorithms. The results of the analyses are reported in Table 4.

In order to compute the theoretical maximal limit of the intraspecific diversity (using a coalescent model) in *Pleurobranchus*, the automatic barcode gap discovery (ABGD) method (Puillandre *et al*., 2012) was implemented for *COI* sequence data. ABGD identifies in the entire distribution of pairwise distances which gap (superior to the maximal limit of the intraspecific diversity) potentially corresponds to the so-called 'Barcoding gap', a hypothetical limit between intra- and interspecific diversity (Puillandre *et al*., 2012). Inference of the limit and gap detection is then
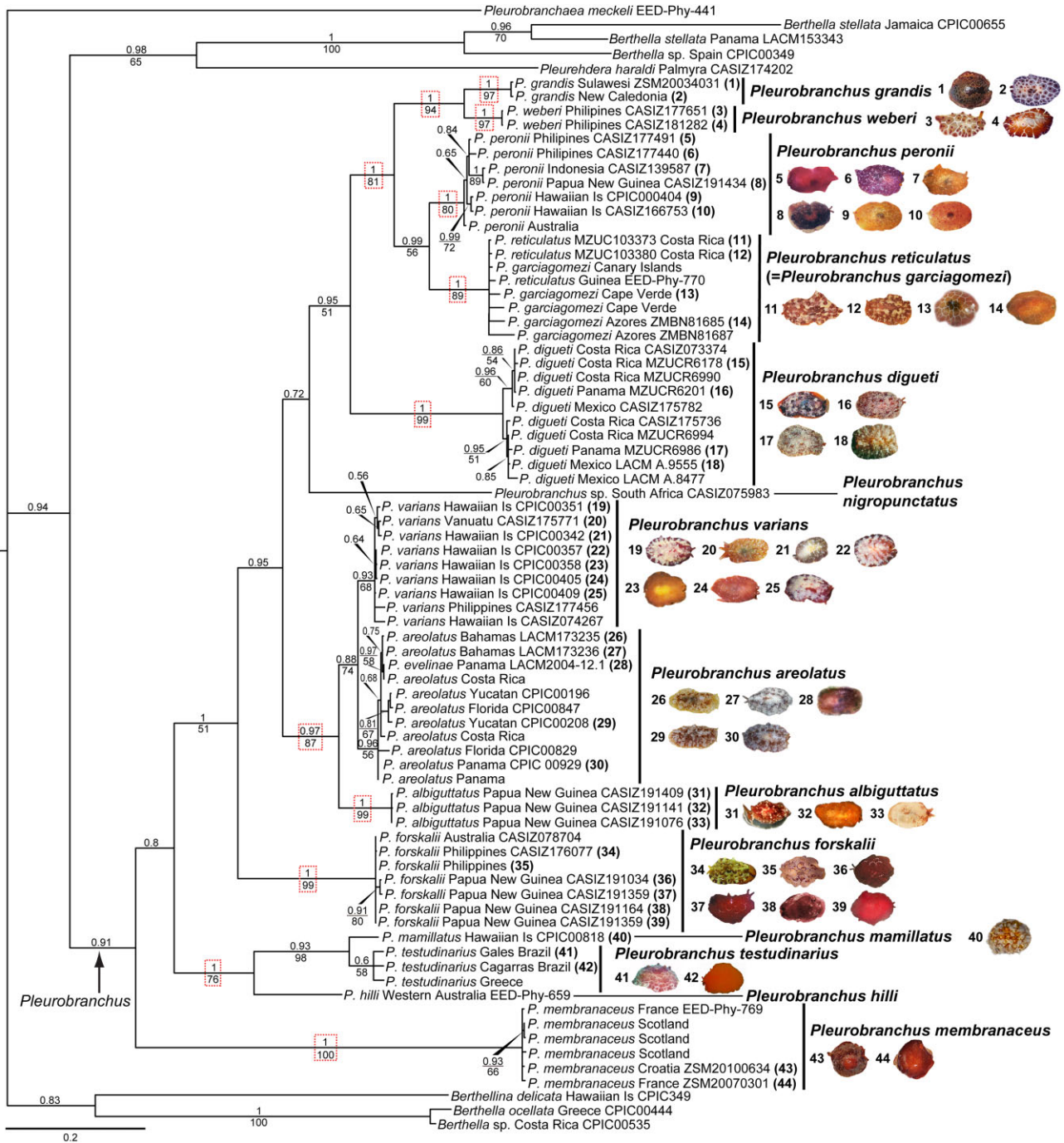
Figure 1. Bayesian consensus phylogenetic tree of *cytochrome c oxidase I*, *histone 3*, and *16S* rDNA genes concatenated for *Pleurobranchus*. Posterior probabilities and bootstrap values from the maximum likelihood analysis are presented for each clade above and below each branch, respectively. Well-supported clades are indicated with a red square around the support values. Bold numbers following the species names indicate the specimen illustrated on the right side of each branch.

recursively applied to previously obtained groups to get finer partitions until there is no further partitioning. The online version of the software (http://wwwabi.snv.jussieu.fr/public/abgd/) was used to analyse the *COI* and *16S* data sets. MEGA 4.0 (Tamura *et al.*, 2007) was used to build the distance matrices for *COI* and *16S* using a Tamura Nei model. The data were analysed using the two available models: Jukes–Cantor

Table 3. Summary of each data set used for analysis with the best-fit evolutionary models and estimated parameters

Parameters	*COI*	*H3*	*16S*
No. of specimens used in the study	67	64	22
No. of included characters	663	328	466
Best-fit model	GTR + I + G	GTR + I + G	HKY + G
Frequency A	0.2773	0.2298	0.3388
Frequency C	0.1042	0.3061	0.1302
Frequency G	0.1623	0.2605	0.1961
Frequency T	0.4561	0.2036	0.3349
R-matrix [A-C]	0.0000	2149277.5	–
R-matrix [A-G]	34.7806	10929979.0	–
R-matrix [A-T]	1.6842	4467196.5	–
R-matrix [C-G]	7.8775	1261227.25	–
R-matrix [C-T]	66.3054	18705996.0	–
R-matrix [G-T]	1.0000	1.0000	–
Γ shape (G)	0.5933	2.4256	–
Proportion of invariable sites (I)	0.5450	0.7014	0

COI, cytochrome c oxidase I; H3, histone 3; HKY, Hasegawa Kishino Yano; GTR, general time reversible.

Table 4. Results from r8s molecular clock analyses, including mean and SD for divergences (in millions of years) for *Pleurobranchus*, using different methods and algorithms. Node codes (letters) are labelled in Figure 34

Node	Langley–Fitch/local molecular clock	Langley–Fitch/Powell	Langley–Fitch/quasi-Newton	Langley–Fitch/truncated Newton	Nonparametric rate smoothing/Powell	Penalized likelihood/Powell	Penalized likelihood/truncated Newton
A	9.95	9.95	9.97	9.97	15.53	12.98	15.58
B	7.97	7.97	7.99	7.99	13.17	10.8	13.15
C	6.26	6.26	6.27	6.27	11.14	8.98	11.13
D	4.69	4.69	4.7	4.7	7.82	5.98	7.82
E	3.91	3.91	3.91	3.91	5.88	4.32	5.89
F	3.55	3.55	3.56	3.56	5.03	3.65	5.04
G	2.96	2.96	2.96	2.96	3.91	2.8	3.92
H	2.18	2.18	2.18	2.18	2.73	1.92	2.73
I	0.89	0.89	0.89	0.89	1.02	0.71	1.02
J	1.42	1.42	1.42	1.42	1.72	1.2	1.72
K	1.39	1.39	1.39	1.39	2.75	1.97	2.76
L	0.69	0.69	0.69	0.69	1.51	1.09	1.54
M	2.87	2.87	2.87	2.87	6.19	4.93	6.18
SD	2.12E-08	2.12E-08	1.71E-09	1.14E-09	5.80E-02	5.29E-02	3.63E-02

(JC69) and Kimura (K80). The program requires two user-specified values: P (prior limit to intraspecific diversity) and X (proxy for minimum gap width). To evaluate the effect on the data sets, X values from 0.1 to 5 were tested and the maximum Pmax value was extended from 0.1 to 0.2 (as in Jörger *et al*., 2012).

MORPHOLOGICAL EXAMINATION

In order to obtain the maximum support for our species determinations, photographs of each specimen were compared to determine the extent of variation in external morphology and colour patterns. We then dissected one to three specimens of all the species examined. The shell of each specimen was dissected and the surrounding tissue removed manually. The buccal mass of each specimen was then removed and the tissue surrounding the jaws and radula was dissolved using 10% sodium hydroxide (NaOH). Shells, jaws, and radulae were rinsed in water, dried, mounted, and sputter-coated for examination with a scanning electron microscope (SEM) Hitachi S-3000N at the Natural History

Museum of Los Angeles County. The features of the reproductive system of all species available were examined and drawn using a dissecting microscope Nikon SMZ-100 with the aid of a *camera lucida* attachment. The reproductive morphology was illustrated as observed; however, several of the specimens dissected were small or poorly preserved and some reproductive organs and connections were difficult to observe.

TYPE MATERIAL

An effort was made to determine the location of the type specimens of all the species of *Pleurobranchus* deposited in museums and other research institutions from all over the world. The institutional abbreviations are as follows: BMNH, The Natural History Museum, London; USNM, Natural History Museum, Smithsonian Institution; MNHN, Muséum National d'Histoire Naturelle, Paris; ZMUC, Zoologisk Museum, Københavns Universitet, Copenhagen; ANSP, Academy of Natural Sciences of Drexel University, Philadelphia; MCZ, Museum of Comparative Zoology at Harvard University; BPBM, Bernice Pauahi Bishop Museum, Honolulu; NL, Naturalis, Leyden; MNCN, Museo Nacional de Ciencias Naturales, Madrid; MCHN, Museo Civico di Storia Naturale di Genova; AM, Australian Museum, Sydney; TAU, Tel Aviv University; IOUSP, Instituto Oceanográfico da Universidade de São Paulo. Some type specimens were examined morphologically when it was necessary to solve outstanding taxonomic problems.

RESULTS

MOLECULAR ANALYSES

The saturation analyses showed insignificant levels of saturation for *H3* and *COI* [Index of substitution saturation (Iss) < Index of substitution saturation critical (Iss.c), $P = 0.000$] even when the third codon positions of these two genes were analysed independently. *16S* has some saturation (Iss < Iss.c, $P = 0.6629$), but it was within the limits of usefulness for phylogenetic analyses. The ILD test showed no significant conflicting signals amongst *H3*, *COI*, and *16S* ($P = 0.94$), *COI* and *H3* ($P = 0.58$), *COI* and *16S* ($P = 0.99$), or *H3* and *16S* ($P = 0.97$).

The Bayesian and maximum likelihood analyses of the concatenated data set (*H3 + COI + 16S*) (Fig. 1), as well as the discrete mitochondrial (*COI*, *16S*) and nuclear (*H3*) gene analyses (Appendix S1), resulted in largely congruent trees. The values given below were obtained from the concatenated data set analyses; values from the single gene analyses are available in Appendix S1. None of the analyses recovered *Pleurobranchus* as a monophyletic group as support values are low: posterior probability (PP) = 0.91, bootstrap support value (BS) < 50. Within *Pleurobranchus*, *P. membranaceus* is monophyletic (PP = 1, BS = 100) and appears to be one of the most basal species of *Pleurobranchus*, although the basal topology of the tree is unresolved owing to poor support. Amongst all other species of *Pleurobranchus*, a clade containing *P. hilli*, *P. mamillatus*, and *P. testudinarius* (PP = 1, BS = 76) is sister to the rest of the species, which form a monophyletic group in the Bayesian tree (PP = 1, BS = 51). *Pleurobranchus forskalii* (PP = 1, BS = 99) comes out as sister to the remaining species of *Pleurobranchus*, which are also monophyletic in the Bayesian tree (PP = 0.95). This clade is further divided into two reciprocally monophyletic clades, the first (PP = 0.97, BS = 87) containing *P. albiguttatus* from the Indo-Pacific (PP = 1, BS = 99), which is sister to a poorly supported clade (PP = 0.88, BS = 74) containing specimens identified as *Pleurobranchus varians* from the Central Pacific (PP = 0.93, BS = 68) and *P. areolatus* from the Caribbean (PP = 0.96, BS = 56). In the second clade, one unidentified *Pleurobranchus* sp. (CASIZ075983) from South Africa comes out as sister to the rest, including specimens identified as *P. areolatus* from the eastern Pacific, here called *Pleurobranchus digueti* (PP = 1, BS = 99). *Pleurobranchus digueti* is sister to a clade containing two more reciprocally monophyletic clades (PP = 1, BS = 81). The first of these (PP = 0.99, BS = 56) contains *P. peronii* from the Indo-Pacific (PP = 1, BS = 80) and a well-supported subclade containing two Brazilian specimens originally identified as *P. areolatus* together with *P. reticulatus* from Guinea and *P. garciagomezi* from the eastern Atlantic (PP = 1, BS = 89). There is no structure within this subclade. The second clade, sister to *P. peronii* + *P. reticulatus* + *P. garciagomezi*, is also well supported (PP = 1, BS = 94), and contains specimens identified as *P. weberi* (PP = 1, BS = 97) and *P. grandis* from the Indo-Pacific (PP = 1, BS = 97).

In all of the ABGD analyses specimens identified as *P. digueti*, *P. forskalii*, *P. mamillatus*, *P. hilli*, and *P. membranaceus* were all recovered as individual groups, whereas those identified as *P. varians*, *P. areolatus*, and *P. albiguttatus* were recovered as a single group. In all analyses conducted using Jukes–Cantor distances, specimens of *P. peronii* were recovered as a single group, and specimens identified as *P. garciagomezi* and *P. reticulatus* were grouped together. In the analyses conducted using Kimura distances, individuals identified as *P. peronii*, *P. garciagomezi*, and *P. reticulatus* were recovered in a single group. In the majority of analyses, individuals identified as *P. weberi* and *P. grandis* were separated into separate groupings, with the exception of two analyses (using Kimura distances, the first using all default parameters except X = 0.1 and the second using all default parameters except $P = 0.2$), where *P. weberi* and *P. grandis* were recovered as a single group.

The r8s analysis (Table 4) indicated that three clades of *Pleurobranchus* diverged around 9.5 Mya, one

splitting further into *P. mamillatus* and *P. hilli* (6.31 Mya). *Pleurobranchus forskalii* diverged from the largest *Pleurobranchus* clade around 7.88 Mya. Following that, this clade split 6.81 Mya into two clades, one containing *P. varians* and the other *P. grandis*. The next split was the divergence of *P. digueti* from the *P. grandis* clade 5.28 Mya, followed by the split of the *P. grandis*/*P. weberi* clade from the *P. reticulatus*/ *P. peronii* clade 4.16 Mya. *Pleurobranchus reticulatus* and *P. peronii* then diverged 3.02 Mya. Finally, *P. albiguttatus* split from the *P. varians*/*P. areolatus* clade 4.35 Mya, followed by the *P. varians*/*P. areolatus* split 3.10 Mya.

SYSTEMATICS

Based on the molecular data and analyses, 14 species of *Pleurobranchus* are recognized as valid. In this section, brief descriptions of these species as well as a diagnosis of *Pleurobranchus* are provided in order to facilitate species identification and future work. The species descriptions are organized chronologically by original description date to simplify reading.

PLEUROBRANCHUS CUVIER, 1804

Diagnosis: Nudipleura side-gilled slugs with a tuberculate mantle and gill rachis, cleft anterior border of the mantle, and flaps surrounding the genital aperture of mature adults. Rhinophores involute and basally joined together, which pulsate regularly in living animals. Penial gland absent, radular teeth typically simple and hamate, jaws composed of denticulate elements. All species with an oval body, mantle notched anteriorly, and a slightly protruding oral veil. Bipinnate gill not visible from dorsal side and covered by mantle, placed on the right-hand side of the body and united with the body for the majority of its length.

Remarks: The first comprehensive taxonomic review of *Pleurobranchus* and closely related taxa was conducted by Bergh (1897). This was followed by two monographical revisions by Vayssière (1898, 1901) who divided *Pleurobranchus* into several genus-level taxa (*Pleurobranchus sensu stricto*, *Oscanius* Leach in Gray, 1847, *Susania* Gray, 1957, *Oscaniella* Bergh, 1897) based on one or a few traits. Willan (1987) synonymized these taxa with *Pleurobranchus*, and this is currently accepted by most authors. The diagnosis above is based on Willan's (1987) revision, which provided an updated diagnosis of *Pleurobranchus* based on a phylogenetic analysis.

PLEUROBRANCHUS PERONII CUVIER, 1804
(FIGS 2A–H, 3B, F, 4, 5, 6)

Pleurobranchus peronii Cuvier, 1804: 266–276, figs 1–6 (type locality: 'Mer des Indes' [Indian Ocean])
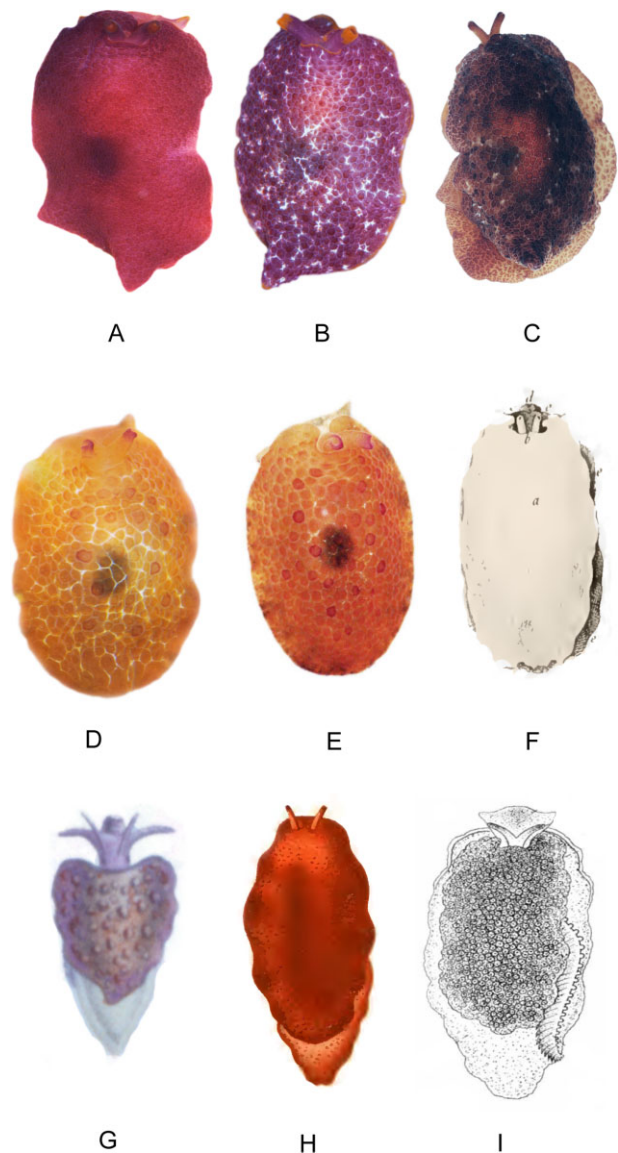

Figure 2. Colour variation in *Pleurobranchus peronii*. A, *P. peronii* from Luzon, Philippines (CASIZ177491). B, *P. peronii* from Luzon, Philippines (CASIZ177440). C, *P. peronii* from Madang, Papua New Guinea (CASIZ191434). D, *P. peronii* from the Hawaiian Islands (CPIC00404). E, *P. peronii* from the Hawaiian Islands (CASIZ166753). F, *P. peronii*, original description illustration from the Indian Ocean (Cuvier, 1804). G, *Pleurobranchus cornutus*, original description illustration from Indonesia (Quoy & Gaimard, 1832–1833). H, *Oscaniella purpurea*, illustration by Bergh (1905) from East Timor. I, *Pleurobranchus hirasei*, original description illustration from Japan (Baba, 1971).

Pleurobranchus cornutus Quoy & Gaimard, 1832–1833: 298–299, pl. 22, figs 20–24 (type locality: Bay of Ambon, Ambon Island, Indonesia) **syn. nov.**
Pleurobranchus ovalis Pease, 1868: 79, pl. 9, fig. 3 (type locality: Tahiti)
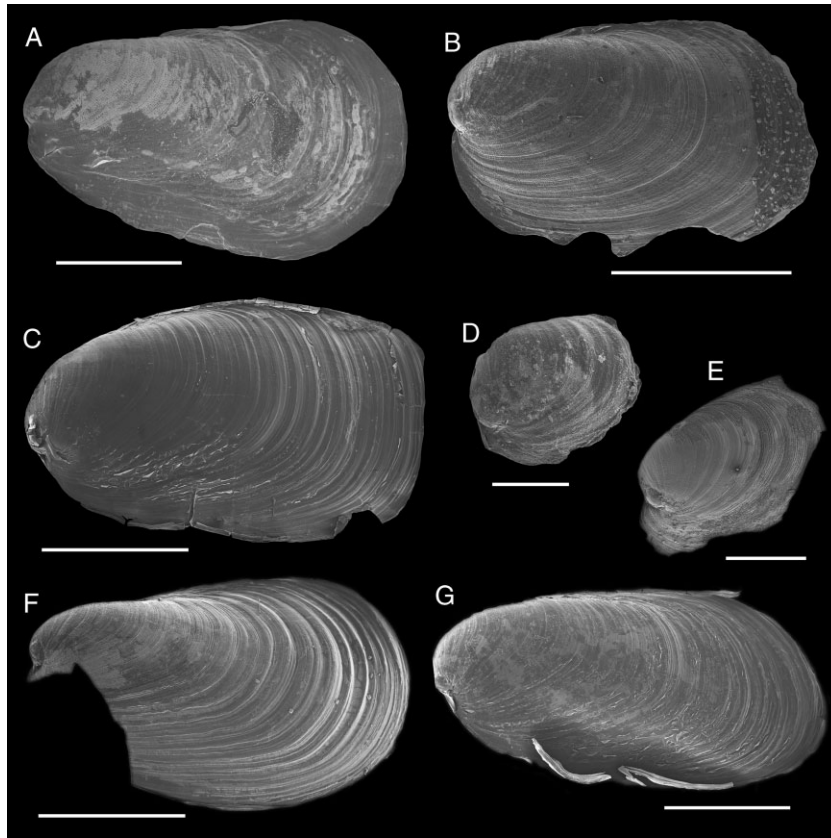
Figure 3. Scanning electron micrographs of shells. A, *Pleurobranchus varians* from the Hawaiian Islands (CPIC00351), scale bar = 1 mm. B, *Pleurobranchus peronii* from the Hawaiian Islands (CPIC00404), scale bar = 2 mm. C, *Pleurobranchus mamillatus* from the Hawaiian Islands (CPIC00818), scale bar = 2 mm. D, *P. varians* from the Hawaiian Islands (CPIC00358), broken, only apex illustrated, scale bar = 500 µm. E, *Pleurobranchus weberi* from Luzon, Philippines (CASIZ177651), scale bar = 1 mm. F, *P. peronii* from Luzon, Philippines (CASIZ177491), scale bar = 2 mm. G, *Pleurobranchus forskalii* from the Great Barrier Reef, Australia (CASIZ078704), scale bar = 2 mm.

Pleurobranchus giardi Vayssière, 1896b: 354–356 (type locality: Camiguin, Luzon, Philippines) **syn. nov.**

Oscaniella purpurea Bergh, 1897: 95–99, pl. 8, figs 28–39 (type locality: Philippines).

Pleurobranchus winckworthi White, 1946: 52–54, figs 1–3 (type locality: Karachi, Pakistan)

Pleurobranchus papillatus Risbec, 1951: 156–157, fig. 15 (type locality: Artillerie, Nouméa, New Caledonia) **syn. nov.**

Pleurobranchus inhacae Macnae, 1962: 174–176, fig. 5 (type locality: Inhaca Island, Mozambique) **syn. nov.**

Pleurobranchus xhosa Macnae, 1962: 176–177, fig. 6 (type locality: Inhaca Island, Mozambique) **syn. nov.**

Pleurobranchus hirasei Baba, 1971: 24–28, pl. 3, figs 1–9 (type locality: Sagami Bay, Japan) **syn. nov.**

Type material: Syntype of *P. peronii* (MNHN), syntype of *P. giardi* (MNHN). Type material of *P. cornutus* and *P. papillatus* is not known to exist, not found at MNHN (Valdés & Héros, 1998). Type material of *P. purpurea* is not known to exist, not found at ZMUC. Type ma-

terial of *P. winckworthi, P. inhacae*, and *P. xhosa* is not known to exist, not found at BMNH. Holotype and four paratypes of *P. hirasei* are known to exist, location unknown. Type material not examined.

Material examined: Luzon, Philippines (CASIZ177491); Luzon, Philippines (CASIZ177440); Madang, Papua New Guinea (CASIZ191434); Hawaiian Islands, USA (CASIZ166753); Kapalua Bay, Maui, Hawaiian Islands, USA (CPIC00404).

Distribution: Known from South Africa, Madagascar, and Tanzania to the western Pacific of Australia, Papua New Guinea, Indonesia, Malaysia, Korea, and Japan to the Hawaiian Islands (Gosliner, Behrens & Valdés, 2008).

External morphology and coloration: Body size up to 60 mm long (Gosliner *et al*., 2008). Rhinophores with multiple horizontal striations along the length. The foot sometimes projects posteriorly from the mantle. Gill rachis tuberculate at the base of the pinnae. Adult
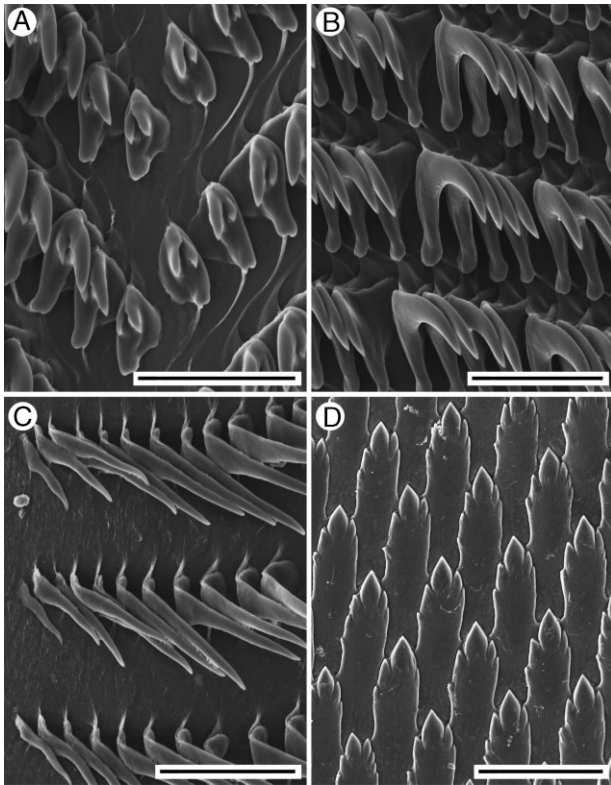
Figure 4. Scanning electron micrographs of the radula and jaws of *Pleurobranchus peronii* from the Hawaiian Islands (CPIC00404). A, innermost lateral teeth, scale bar = 30 μm. B, mid-lateral teeth, scale bar = 30 μm. C, outermost lateral teeth, scale bar = 30 μm. D, jaw elements, scale bar = 50 μm.

tubercles polygonal in shape, larger and more flattened than those in *P. albiguttatus*. Juvenile tubercles conical or hemispherical, getting bigger as the individuals mature. In some specimens, tubercles similar in size, in others smaller in size towards the edge of the mantle.

Background colour orange to dark red in adults and juveniles often a somewhat transparent white. Some tubercles darker; in adults this pigment concentrated at the edges of the tubercle. White, reticulated lines usually found on much of the mantle. Oral veil and rhinophores are the same colour as the mantle and the tips of the rhinophores are often a different colour than that along their length (Fig. 2A–E).

Internal anatomy: Shell oval in shape and the same width along its length. The protoconch is roughly 400 μm, has about one whorl, and lacks any ornamentation (Fig. 3B, F).

Elongated cruciform elements in jaws have a prominent cusp flanked by two to three short denticles on each side (Figs 4D, 5D). Radular formula 43 × 59.0.59 (CPIC 00404) and 54 × 78.0.78 (CASIZ 177491). Radular teeth smooth and hook-shaped and the inner lateral
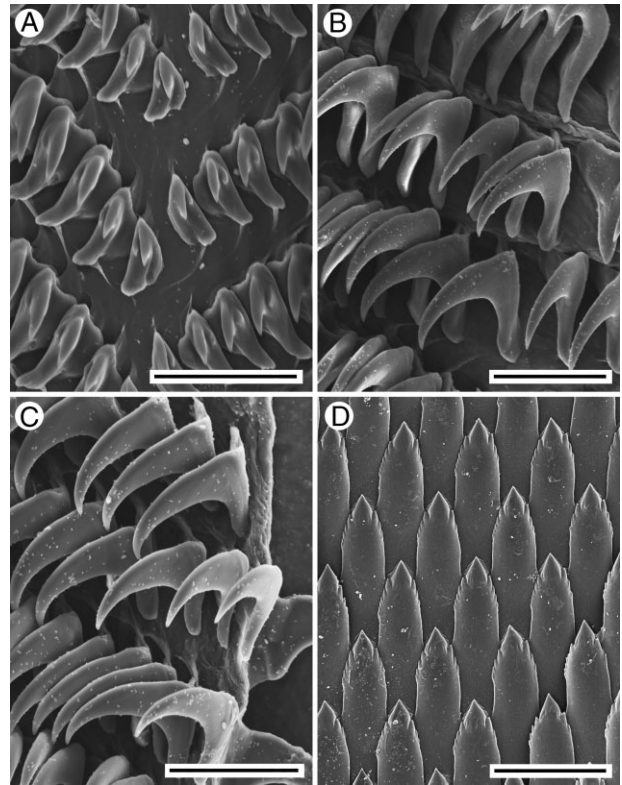

Figure 5. Scanning electron micrographs of the radula and jaws of *Pleurobranchus peronii* from Luzon, Philippines (CASIZ177491). A, innermost lateral teeth, scale bar = 50 μm. B, mid-lateral teeth, scale bar = 50 μm. C, outermost lateral teeth, scale bar = 50 μm. D, jaw elements, scale bar = 100 μm.
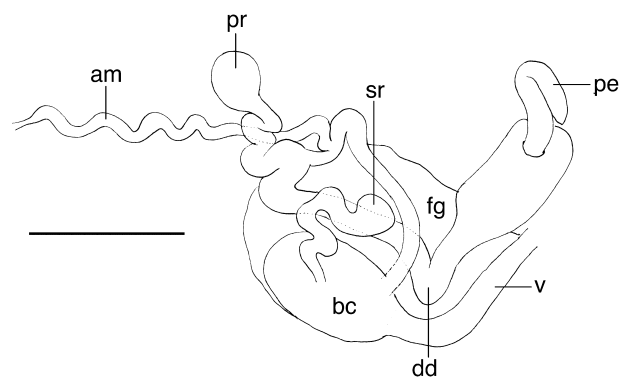
Figure 6. Reproductive system of *Pleurobranchus peronii* from Luzon, Philippines (CASIZ177491), scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland complex; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.

teeth are the shortest. Mid-lateral teeth 2–3× the length of the inner lateral teeth, and outermost lateral teeth 2–3× the length of the mid lateral teeth. (Figs 4A–C, 5A–C).

Reproductive system (Fig. 6) triaulic *sensu* Cervera *et al.* (2000), with the sperm storage organs connected to the female gland complex only at the common genetial atrium. Ampulla simple, muscular, splitting into two ducts, one connecting to the bursa copulatrix and the other to the prostate. No connection with the female gland complex was observed as in Cervera *et al.* (2000) for *P. areolatus*, but this is probably because of the small size of the reproductive system examined. Bursa copulatrix rounded connecting to two ducts; one opens into the vagina and the other to the short, rounded, irregular seminal receptacle. Vaginal duct straight and simple. Prostate long, simple, and connected by the deferent duct to the large and simple penis.

Remarks: Pleurobranchus peronii was originally described and illustrated by Cuvier (1804) and is the type species of *Pleurobranchus*. *Pleurobranchus peronii* differs from similar species of *Pleurobranchus* (such as *P. forskalii* and *P. varians*) by the dark ring of pigment along the edges of some of the polygonal tubercles. The body colour of *P. peronii* ranges from dark purple to red or orange, but the distinctive darker pigment present along some tubercles remains. Additionally, *P. peronii* is distinguishable from other species by having a different colour on the tips of the rhinophores than at the base.

A review of the literature revealed that several species of *Pleurobranchus* are synonymous with *P. peronii*. These include *Pleurobranchus cornutus* Quoy & Gaimard, 1832, *Pleurobranchus ovalis* Pease, 1860, *Pleurobranchus giardi* Vayssière, 1896, *Pleurobranchus purpurea* (Bergh, 1897), *Pleurobranchus winckworthi* White, 1946, *Pleurobranchus papillatus* Risbec, 1951, *Pleurobranchus inhacae* Macnae, 1962, *Pleurobranchus xhosa* Macnae, 1962, and *Pleurobranchus hirasei* Baba, 1971 (Fig. 2F–I). *Pleurobranchus purpurea* was synonymized with *P. peronii* by Edmunds & Thompson (1972) and Er. Marcus (1965) owing to similarities in external morphology. Edmunds & Thompson (1972) also synonymized *P. winckworthi* with *P. peronii*. Rudman (1999b) suggested that *P. ovalis* was a colour form of *P. peronii*. Additionally, *P. cornutus*, *P. giardi*, *P. papillatus*, *P. inhacae*, *P. xhosa*, and *P. hirasei* match the description of *P. peronii*, having small, simple tubercles, some of which have darker rings of pigment around the edges.

PLEUROBRANCHUS MEMBRANACEUS (MONTAGU, 1815)
(FIG. 7F, G)

Pleurobranchus tuberculatus Meckel, 1808: 26, 33, pl. 38, figs 33–37, 40 (type locality: Sicily, Italy)
   Lamellaria membranacea Montagu, 1815: 184–186, pl. 12, fig. 3 (type locality: Kingsbridge, Devon, UK)
   ?*Pleurobranchus lesuerii* Blainville, 1825: 470, pl. 43, fig. 2 (type locality: not specified)

?*Pleurobranchus contarinii* Vérany, 1846 (type locality: Gulf of Genoa, Mediterranean Sea)
?*Pleurobranchus denotarisii* Vérany, 1846: 16, 19 (type locality: Gulf of Genoa: Mediterranean Sea)
Gymnotoplax barashi Ev. Marcus, 1977: 418–420, figs 1–5, 8–11 (type locality: Haifa Bay, Mediterranean Sea)

Type material: The type material of *P. tuberculatus* is not known to exist. Types of *P. membranaceus* and *P. lesuerii* are not known to exist, not found at MNHN (Valdés & Héros, 1998). Types of *P. contarinii* and *P. denotarisii* are not known to exist, not found at MCHN. Syntypes of *G. barashi* are not known to exist, not found at TAU (Mienis, 2014).

Distribution: Known from the UK and France in the North Atlantic and Spain, Greece, and Croatia in the Mediterranean (Rudman, 2001).

External morphology and coloration: Description provided in Thompson & Slinn (1959).

Internal anatomy: Description provided in Thompson & Slinn (1959) and Martynov & Schrödl (2009).

Remarks: Pleurobranchus membranaceus was first described and illustrated by Montagu (1815), but Pilsbry (1895) considered this species to be a synonym of *P. tuberculatus* Meckel, 1808. However, a number of more recently published papers have maintained *P. membranaceus* as a valid species and *P. tuberculatus* as a synonym despite the fact that *P. tuberculatus* is an older name (Edmunds, 1968; Thompson, 1969, 1970, 1976, 1983; Cervera, Cattaneo-Vietti & Edmunds, 1996; Cervera *et al.*, 2000, 2006; Martynov & Schrödl, 2009; Göbbeler & Klussmann-Kolb, 2010). The similarities in morphology between the two species include the presence of reddish-brown, small tubercles that irregularly cover the mantle, and a foot that extends well beyond the posterior edge of the mantle (Fig. 7F–G). Given these similarities, we agree that these two species are synonyms. However, despite the fact that *P. membranaceus* is currently used as the valid name for this species, we are unable to propose a Reversal of Precedence, as stated in the International Code of Zoological Nomenclature (ICZN, 1999) because Pruvot-Fol (1954) used the name *P. tuberculatus* as valid. Therefore, this case will have to be referred to the International Commission on Zoological Nomenclature at a later date.

Other species names also considered to be synonyms of *P. membranaceus* are *P. lesuerii* Blainville, 1825, *P. contarinii* Vérany, 1846, and *P. denotarisii* Vérany, 1846 by Pilsbry (1895), and *Gymnotoplax barashi* Ev. Marcus, 1977 by Ev. Marcus (1984). We could not confirm these synonymies owing to incomplete original descriptions with the exception of *G. barashi*, which was synonymized with *P. membranaceus* by Willan (1978).
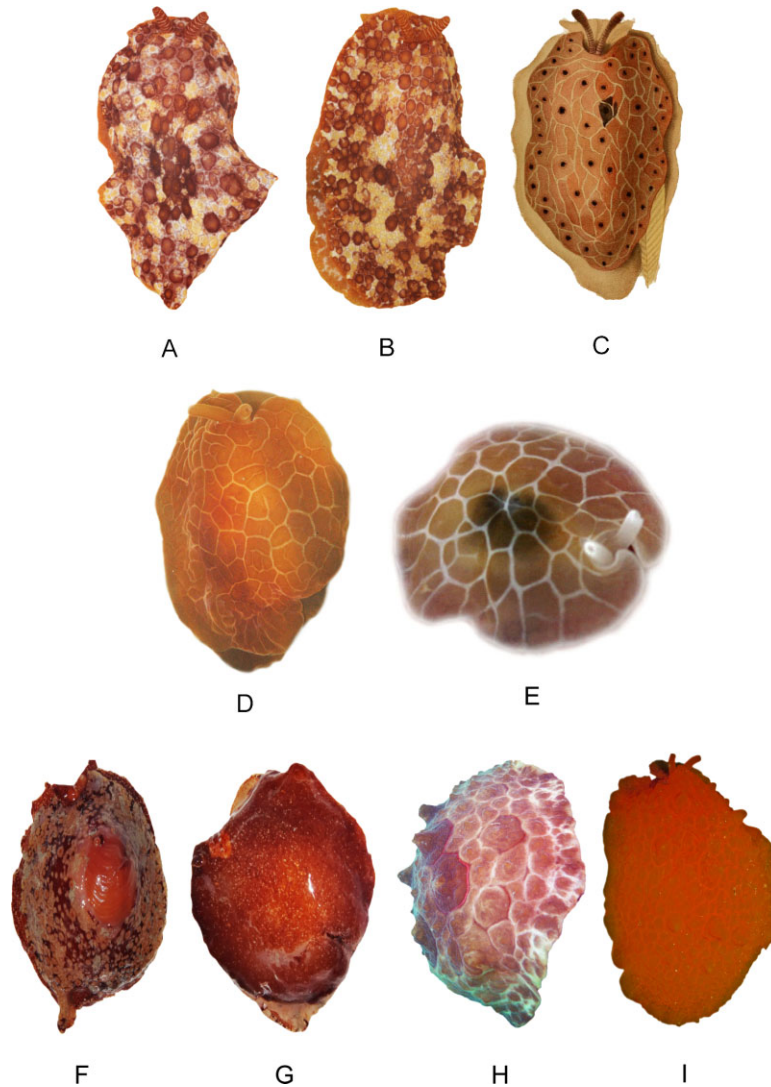
Figure 7. Colour variation in *Pleurobranchus reticulatus*, *Pleurobranchus membranaceus*, and *Pleurobranchus testudinarius*. A, *P. reticulatus* from Brazil (MZSP103373). B, *P. reticulatus* from Brazil (MZSP103380). C, *P. reticulatus*, original description illustration from Principe Island (Rang, 1832). D, *Pleurobranchus garciagomezi* from Azores (ZMBN81685). E, *P. garciagomezi* from Cape Verde (no voucher no.). F, *P. membranaceus* (ZSM20100634). G, *P. membranaceus* from France (ZSM20070301). H, *P. testudinarius* from Santa Catarina, Brazil (photo by Edson Júnior). I, *P. testudinarius* from Rio de Janeiro, Brazil (photo by Fernando Moraes).

PLEUROBRANCHUS FORSKALII RÜPPELL & LEUCKART, 1828 (FIGS 8–11)

'Lepus marinus' Forsskål, 1776 (type locality: not specified)

Pleurobranchus forskalii Rüppell & Leuckart, 1828: 18–20, pl. 5, fig. 2A, B (type locality: Red Sea)

Pleurobranchus perrieri Vayssière, 1896a: 126–128, pl. 4, figs 2–4 (type locality: not specified; cited from Philippines; Ambon, Moluccas, Indonesia; Tahiti) **syn. nov.**

Oscanius semperi Vayssière, 1896a: 134, 135, pl. 4, fig. 1 (type locality: Philippines) **syn. nov.**

?*Susania karachiensis* White, 1946: 55, 56, figs 8–10, pl. 5, fig. 7 (type locality: Karachi, Pakistan)

?*Susania ceylonica* White, 1948: fig. 2 (type locality: 'Cheval Parr, Ceylon' [Sri Lanka])

Type material: Sixteen syntypes of *P. perrieri* (MNHN). The type material of *P. semperi* is not known to exist, not found at MNHN (Valdés & Héros, 1998). Types of *P. karachiensis* and *P. ceylonicus* are not known to exist, not found at BMNH. No type material examined.

Material examined: Philippines (voucher number unknown); Madang, Papua New Guinea (CASIZ191034);
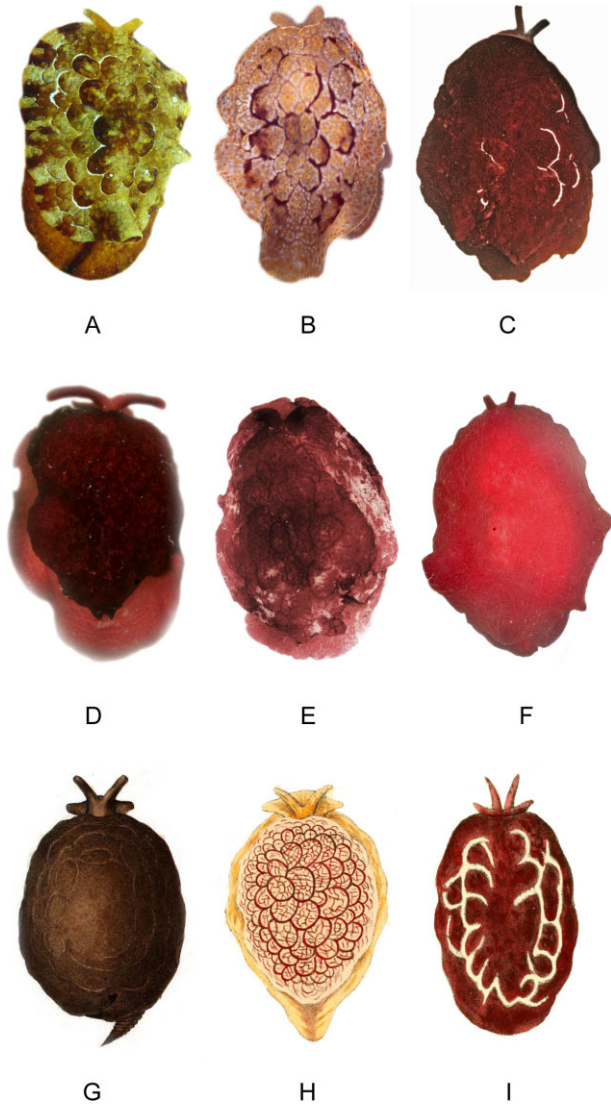
Figure 8. Colour variation in *Pleurobranchus forskalii*. A, *P. forskalii* from the Philippines (CASIZ176077). B, *P. forskalii* from the Philippines (no voucher no.). C, *P. forskalii* from Madang, Papua New Guinea (CASIZ191034). D, *P. forskalii* from Madang, Papua New Guinea (CASIZ191359). E, *P. forskalii* from Madang, Papua New Guinea (CASIZ191164). F, *P. forskalii* from Madang, Papua New Guinea (CASIZ191300). G, *P. forskalii*, original description illustration from the Red Sea (Rüppell & Leuckart, 1828). H, *Pleurobranchus semperi*, original description illustration from the Philippines (Vayssière, 1896a). I, *Pleurobranchus perrieri*, original description illustration from the tropical Indo-Pacific (Vayssière, 1896a).

Madang, Papua New Guinea (CASIZ191359); Philippines (CASIZ176077); Madang, Papua New Guinea (CASIZ191164); Madang, Papua New Guinea (CASIZ191300); Great Barrier Reef, Australia (CASIZ078704).
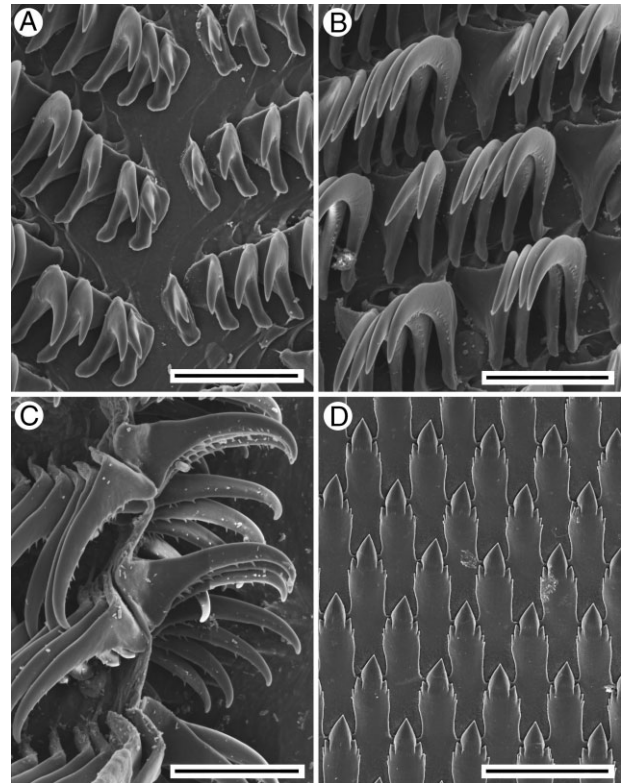

Figure 9. Scanning electron micrographs of the radula and jaws of *Pleurobranchus forskalii* from the Great Barrier Reef, Australia (CASIZ078704). A, innermost lateral teeth, scale bar = 50 μm. B, mid-lateral teeth, scale bar = 50 μm. C, outermost lateral teeth, scale bar = 50 μm. D, jaw elements, scale bar = 100 μm.

Distribution: Known from Tanzania, the Red Sea, Australia, Fiji, New Guinea, Indonesia, Philippines, Guam, Japan, and Australia (Gosliner *et al.*, 2008).

External morphology: Body size up to 300 mm long (Gosliner *et al.*, 2008). Rhinophores with multiple horizontal striations along the length. Posterior end of the foot projecting from the mantle in some specimens. Gill rachis tuberculate at the base of the pinnae and axes of the pinnae tuberculate. Broad, compound tubercles in adults usually outlined in coloured arcs.

Background colour in mature animals light, mottled orange-brown or dark red. Tubercles outlined with white arcs in dark animals and dark arcs in light animals. Tubercles not outlined in some specimens. Oral veil and rhinophores same colour as the mantle and white-tipped in juveniles (Fig. 8A–F).

Internal anatomy: Shell oval in shape and narrower at the anterior end than the posterior. Protoconch roughly 600 μm, has about one whorl and lacks any ornamentation.

Elongated cruciform elements in jaws have a prominent cusp flanked by two to three short denticles on
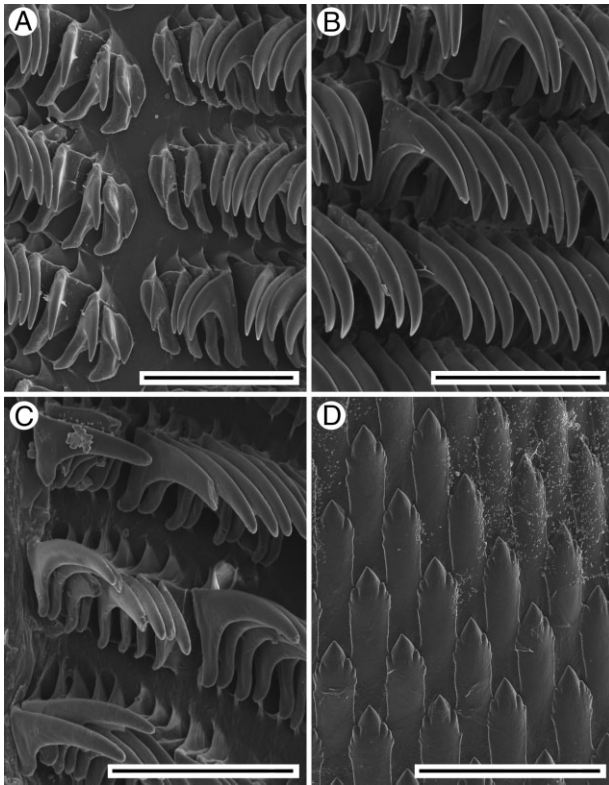
Figure 10. Scanning electron micrographs of the radula and jaws of *Pleurobranchus forskalii* from the Philippines (CASIZ176077). A, innermost lateral teeth, scale bar = 100 μm. B, mid-lateral teeth, scale bar = 100 μm. C, outermost lateral teeth, scale bar = 100 μm. D, jaw elements, scale bar = 200 μm.

each side (Figs 9D, 10D). Radular formula 37 × 87.0.87 (CASIZ 078704) and 67 × 123.0.123 (CASIZ 176077). Radular teeth smooth and hook-shaped, innermost lateral teeth the shortest. Fine denticulations can be observed in some teeth (Fig. 9B) but this appears to be a preservation artefact. Mid-lateral teeth roughly 2–3× the length of the inner teeth and outermost lateral teeth 1–2× larger than the mid-lateral teeth (Figs 9A–C, 10A–C).

Reproductive system (Fig. 11) triaulic. Ampulla long, thick, convoluted muscular duct connecting directly into the prostate. Prostate large and rounded, narrowing into a long deferent duct, progressively becoming thin and convoluted. The prostate also connects to the female gonoduct by a duct that connects first to the female gland complex, then to the vagina, and finally to seminal receptacle and bursa copulatrix. Deferent duct connecting directly to the large penis. Seminal receptacle large, elongate, and irregularly shaped, bursa copulatrix large and oval.

Remarks: Rüppell & Leuckart (1828) described *P. forskalii* from a figure, originally published by Forsskål
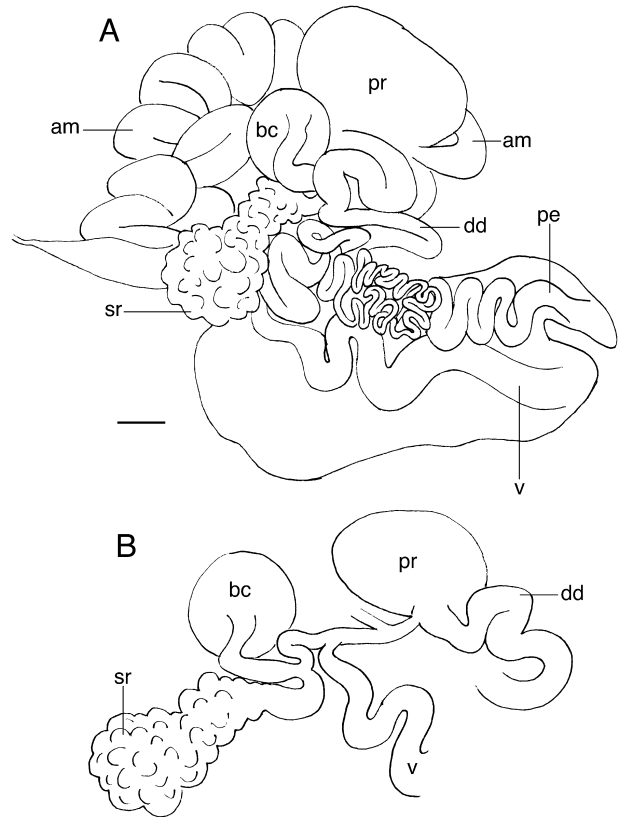

Figure 11. Reproductive system of *Pleurobranchus forskalii* from the Philippines (CASIZ176077). A, reproductive system; B, detail of some reproductive organs. Scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; dd, deferent duct; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.

(1776), labelled 'Lepus marinus', from Massawa, Eritrea (Red Sea). Yaron, Schiøtte & Wium-Andersen (1986) reproduced the original figure in Forsskål (1776), which represents a pleurobranchid, closely resembling the specimens here assigned to *P. forskalii*. Yaron *et al.* (1986) considered that the original description of 'Lepus marinus' is valid as it is accompanied by an indication to a work by Avicenna. However, Rudman (1993) considered 'Lepus marinus' a vernacular name and therefore invalid. Rudman (1993) submitted a petition to the International Commission on Zoological Nomenclature requesting that *P. forskalii* be considered the valid name for this species, as there was currently another species of *Pleurobranchus* described from the Mediterranean with a similar name (*Pleurobranchus forskahli* Delle Chiaje, 1822). In Opinion #1767, the Commission accepted the suppression of *P. forskahli*, placing it as a synonym of *P. testudinarius*, and considered *P. forskalii* a valid species name.

In 1896, Vayssière published two new species of *Pleurobranchus* (*P. perrieri* and *P. semperi*) that we con-

sider to be synonyms of *P. forskalii*. The original description illustrations of *P. perrieri* and *P. semperi* have the distinguishing colour pattern of *P. forskalii*, the opaque white or black circles or semicircles that are often present on the dorsum (Fig. 8G–I). Additionally, *P. karachiensis* and *P. ceylonicus*, described by White (1946, 1948) are probably synonyms of *P. forskalii* as well. *Pleurobranchus karachiensis* was described as having 'large and small tubercles arranged in circles and forming a regular pattern. The colour of the preserved specimen is grey with brown rings that outline the surface of the tubercles.' This matches the description of some specimens of *P. forskalii*, which have opaque black markings that form a semicircle around small tubercles arranged in clusters. *Pleurobranchus ceylonicus* was also described as having a golden-brown body with darker brown tubercles that were each encircled by a bright blue line. In some specimens of *P. forskalii*, the compound tubercles almost seem to be one entity that is often surrounded by an opaque line. In addition, the small size and colour of the specimen is consistent with a juvenile *P. forskalii*. Colour illustrations, however, were not provided for either *P. karachiensis* or *P. ceylonicus*, so we cannot be completely sure of their identity.

PLEUROBRANCHUS MAMILLATUS QUOY & GAIMARD, 1832 (FIGS 3C, 12I, J, 13)

Pleurobranchus mamillatus Quoy & Gaimard, 1832–1833: 294–296, pl. 22, figs 1–6 (type locality: Port Louis, 'Ile de France' [Mauritius])

?*Pleurobranchus moebii* Vayssière, 1896a: 128–130, pl. 4, figs 5, 6 (type locality: Querimba Islands, Mozambique)

Type material: Two syntypes of *P. mamillatus* (MNHN). Type material of *P. moebii* is not known to exist, not found at MNHN (Valdés & Héros, 1998). No type material was examined.

Material examined: Maalaea Bay, Maui, Hawaiian Islands, USA (CPIC00818).

Distribution: Known from Tanzania, South Korea, Japan, and the Hawaiian Islands (Gosliner *et al.*, 2008).

External morphology and coloration: Body size up to 500 mm long (Gosliner *et al.*, 2008). Rhinophores smooth. Foot does not project from the mantle. Gill rachis tuberculate at the base of the pinnae. Tubercles round and weakly compound and become more elongate from the edge of the mantle to the centre of the dorsum. Secondary tubercles flat around the edge of the mantle.

Background colour in juveniles cream to yellow or light brown, and in adults light brown to dark violet. Tubercles partially outlined in bright violet arcs and sometimes spots. Oral veil and rhinophores same colour as the mantle, sometimes with a different pigment at the tips of the rhinophores (Fig. 12I).

Internal anatomy: Shell oval in shape and the same width along its length. Protoconch roughly 250 μm, has about one whorl and lacks any ornamentation (Fig. 3C).

Elongated cruciform elements in jaws have a prominent cusp flanked by two to three short denticles on each side (Fig. 13D). Radular formula 49 × 109.0.109 (CPIC 00818). Radular teeth smooth and hook-shaped and the inner lateral teeth are the shortest. Mid-lateral teeth 4–5× the length of the inner lateral teeth, and outermost lateral teeth roughly the same as the mid-lateral teeth. (Fig. 13A–C).

Remarks: Pleurobranchus mamillatus was originally described as having a series of strikingly elevated tubercles on the mantle, with some smaller tubercles arranged in clusters (Quoy & Gaimard, 1832–1833). Additionally, this species most often has a tan, orange to reddish, or black body colour (Fig. 12J). Both Pilsbry (1895) and Thompson (1970) considered *P. mamillatus* to be a valid species.

One species that may be a potential synonym of *P. mamillatus* is *Pleurobranchus moebii* Vayssière, 1896. In the original description, *P. moebii* is described as having large, protruding papillae (Vayssière, 1896a), which is consistent with the description of *P. mamillatus*. However, as the only illustration provided by the author is that of the shell, this synonymy is difficult to confirm. Additionally, juveniles of *P. forskalii* are commonly confused with juveniles of *P. mamillatus*, although juveniles of *P. forskalii* lack the violet arcs and spots prevalent in *P. mamillatus*.

PLEUROBRANCHUS RETICULATUS RANG, 1832 (FIG. 7A–E)

Pleurobranchus reticulatus Rang, 1832: pl. 1 (type locality: Probably San Antonio Bay, Principe Island, São Tomé, and Principe)

?*Pleurobranchus garciagomezi* Cervera, Cattaneo-Vietti & Edmunds, 1996: 150–156, figs 1–4, pl. 1 (type locality: Albacora Bay, Ilha do Sal, Cape Verde Islands)

Type material: Type material of *P. reticulatus* is not known to exist, not found at MNHN (Valdés & Héros, 1998). Holotype of *P. garciagomezi* (MNCN 15.05/15840) and paratype (MCHN).

Material examined: Saco da Pedra Reef, Alagoas, north-eastern Brazil (MZSP 103373) and Saco da Pedra Reef, Alagoas, north-eastern Brazil (MZSP 103380)

Distribution: Originally described from the Gulf of Guinea, West Africa (Neves *et al.*, 2007), and recorded as *P. areolatus* from Brazil (Marcus, 1976; García, Troncoso & Domínguez, 2002; Padula *et al.*, 2012), Madeira (see Cervera *et al.* 2004), and the Ascension islands (Padula *et al.* 2014b). If the synonymy of
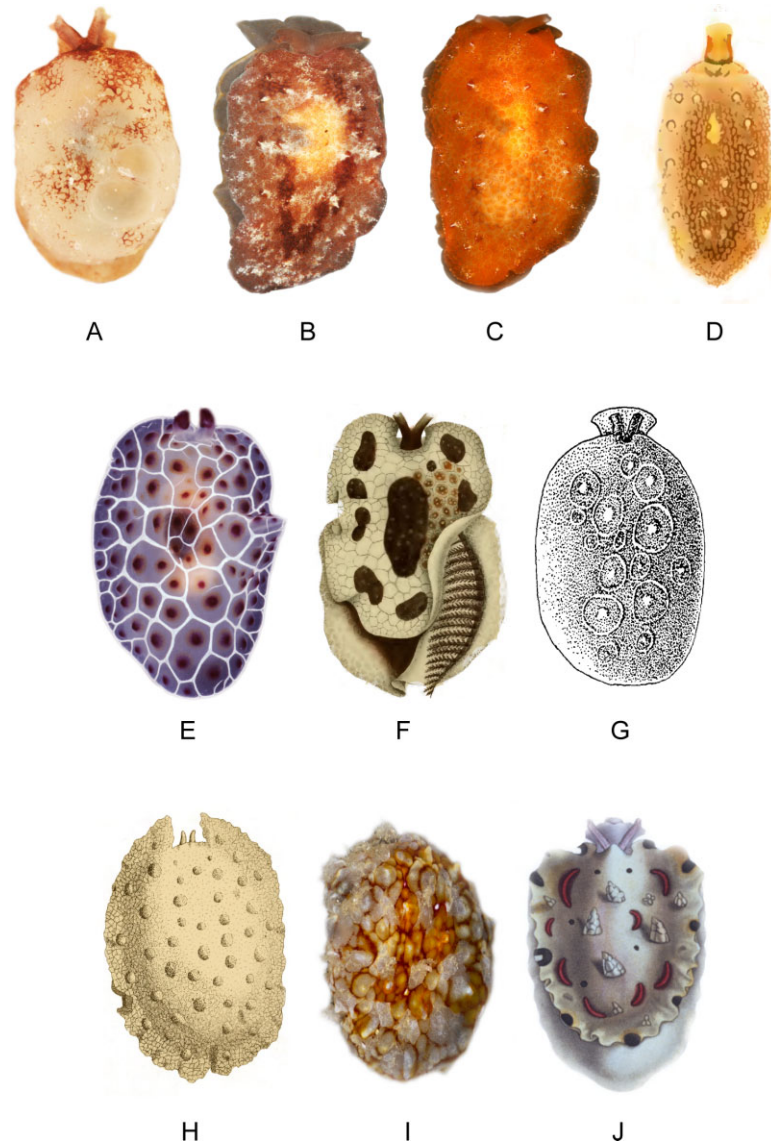
Figure 12. Colour variation in *Pleurobranchus albiguttatus*, *Pleurobranchus grandis*, *Pleurobranchus hilli*, and *Pleurobranchus mamillatus*. A, *P. albiguttatus* from Madang, Papua New Guinea (CASIZ191076). B, *P. albiguttatus* from Madang, Papua New Guinea (CASIZ191409). C, *P. albiguttatus* from Madang, Papua New Guinea (CASIZ191141). D, *P. albiguttatus*, original description illustration from Indonesia (Bergh, 1905). E, *P. grandis*, juvenile from New Caledonia (no voucher no.). F, *P. grandis*, original description illustration from Huahine, French Polynesia (Pease, 1868). G, *Pleurobranchus iouspi*, original description illustration from Brazil (Ev. Marcus, 1984). H, *P. hilli*, original description illustration from New South Wales, Australia (Hedley, 1894). I, *P. mamillatus* from the Hawaiian Islands (CPIC00818). J, *P. mamillatus*, original description illustration from Mauritius (Quoy & Gaimard, 1832–1833).

P. garciagomezi is confirmed, the species is also recorded from the Canary Islands, Cape Verde, and the Azores (Cervera *et al*., 2006; Malaquias *et al*., 2009).

External morphology and coloration: Descriptions provided in Neves *et al*. (2007) and Cervera *et al*. (1996). Photographs of Brazilian specimens provided by García *et al*. (2002), Padula *et al*. (2012), and in the present study (Fig. 7A, B).

Internal anatomy: Descriptions provided in Neves *et al*. (2007) and Cervera *et al*. (1996).

Remarks: Pleurobranchus reticulatus was originally described and illustrated by Rang (1832). The species was then redescribed by Neves *et al*. (2007), where they included data on the anatomy of the reproductive system and the development of colour pattern in this species. This species can be recognized by the thinner white
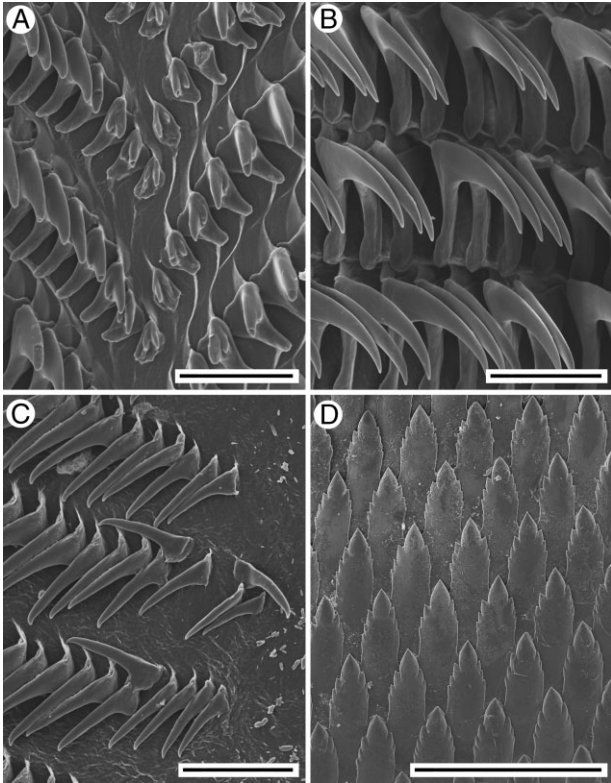
Figure 13. Scanning electron micrographs of the radula and jaws of *Pleurobranchus mamillatus* from the Hawaiian Islands (CPIC00818). A, innermost lateral teeth, scale bar = 50 µm. B, mid-lateral teeth, scale bar = 50 µm. C, outermost lateral teeth, scale bar = 50 µm. D, jaw elements, scale bar = 200 µm.

reticulations on the mantle and the presence of dark tubercles inside the polygonal areas in the adults (Fig. 7C).

Cervera *et al*. (1996) described *P. garciagomezi* and separated this species morphologically from *P. reticulatus* by the thicker white reticulations on the mantle and the lack of dark tubercles in the adult specimens. The type locality of *P. garciagomezi* is Cape Verde, but the species has been subsequently reported from the Azores, the Canary Islands, and Madeira (Cervera *et al*., 1996; Rudman, 2000). Molecular data obtained in the present study from specimens collected in Cape Verde, the Azores, and the Canary Islands (Fig. 7D, E) are genetically similar to a Guinean specimen identified as *P. reticulatus* (FJ917498) by Göbbeler & Klussmann-Kolb (2010) and to specimens collected in Brazil, originally identified as *P. areolatus*. As there are no photographs available of the *P. reticulatus* specimen from the Canary Islands, it is not possible to confirm it belongs to this species; however, the specimens from Brazil are externally clearly distinct from other specimens of *P. garciagomezi* collected to date, with a reddish mantle (Fig. 7A–B) lacking

the characteristic thicker white reticulations. This suggests that this species, as do many other species of *Pleurobranchus*, may display substantial chromatic variation. Both the phylogenetic and ABGD analyses indicated that *P. garciagomezi* is not distinct from the specimens assigned to *P. reticulatus*, but we are not definitely synonymizing these two species because we are not confident about the correct identification of the specimen of *P. reticulatus* from the Canary Islands. Further evidence is necessary to fully determine the relationship between *P. garciagomezi* and *P. reticulatus*.

PLEUROBRANCHUS TESTUDINARIUS CANTRAINE, 1835 (FIG. 7H, I)

Pleurobranchus testudinarius Cantraine, 1835: 385 (type locality: Naples, Italy)

?Pleurobranchus iouspi Ev. Marcus, 1984: 68–70, figs 57–61 (type locality: São Paulo, Brazil)

Type material: Type material of *P. testudinarius* is not known to exist, not found at MNHN (Valdés & Héros, 1998). According to Ev. Marcus (1984), type of *P. iouspi* was deposited at IOUSP but it is no longer there or at MZUSP (M. Petti & D. Cavallari, pers. comm.).

Distribution: Known from the Mediterranean Sea and Brazil (Marcus, 1971; Rudman, 1999a; Cunha *et al*. 2014).

External morphology and coloration: Description provided in Cattaneo-Vietti (1986). In addition, Brazilian specimens can be completely orange as well (Fig. 7I).

Internal anatomy: Description provided in Cattaneo-Vietti (1986) and Martynov & Schrödl (2009).

Remarks: Cantraine (1835) originally described *P. testudinarius* from the Mediterranean, and it has been deemed valid by Pilsbry (1895), Bergh (1897), and Cervera *et al*. (1996). The species was later recorded from Brazil based on a single specimen (Marcus, 1971). *Pleurobranchus testudinarius* can be distinguished by large, polygonal tubercles present on the mantle that 'occupy a mesh of a rose-carmine network' (Pilsbry, 1895).

Although *P. iouspi* was described and illustrated from São Paulo, Brazil, by Ev. Marcus (1984) and was determined to be valid by Cervera *et al*. (1996), very few accounts of this species exist in the literature. Morphologically, *P. iouspi* is very similar to *P. testudinarius* described by Cantraine (1835) from the Mediterranean. Both have large, complex tubercles that cover the mantle, although the colour patterns could not be compared because *P. iouspi* was described from a preserved specimen. Our molecular data indicate that these two species are genetically identical. However, we have only nuclear data from all three specimens, two from Brazil and one from the Mediterranean. As such, and

because of the morphological similarity between these two species, it is very probable that they are synonyms, but this needs to be confirmed by additional evidence.

PLEUROBRANCHUS VARIANS PEASE, 1860
(FIGS 3A, D, 14A–G, 15–17)

Pleurobranchus varians Pease, 1860: 25 (type locality: 'Sandwich Islands' [Hawaiian Islands, USA])

Type material: Type material of *P. varians* is not known to exist, not found at BMNH.

Material examined: Honokeana Bay, Maui, Hawaiian Islands, USA (CPIC00351); Maliko Bay, Maui, Hawaiian Islands, USA (CPIC00358); Honokeana Bay, Maui, Hawaiian Islands, USA (CPIC00342); Maliko Bay, Maui, Hawaiian Islands, USA (CPIC00357); Kapalua Bay, Maui, Hawaiian Islands, USA (CPIC00405); Honokeana Bay, Maui, Hawaiian Islands, USA (CPIC00409); Espiritu Santo Island, Vanuatu (CASIZ175771).

Distribution: Known from the Hawaiian Islands and Vanuatu.
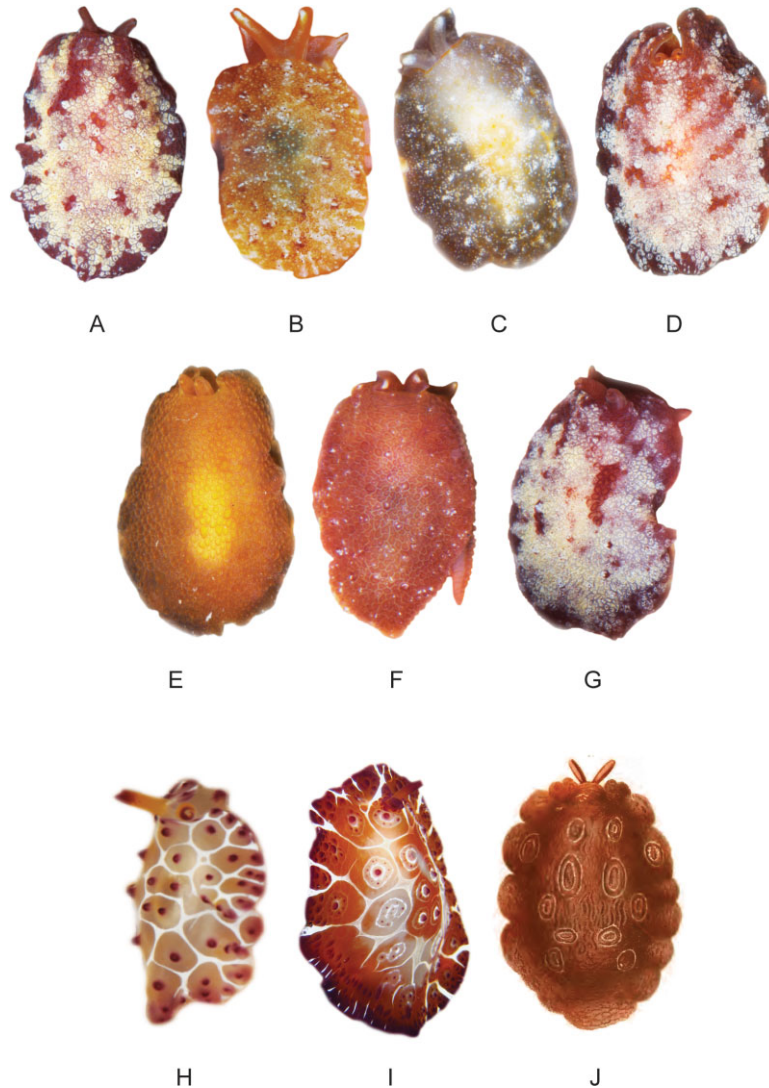
Figure 14. Colour variation in *Pleurobranchus varians* and *Pleurobranchus weberi*. A, *P. varians* from the Hawaiian Islands (CPIC00351). B, *P. varians* from Vanuatu (CASIZ175771). C, *P. varians* juvenile from the Hawaiian Islands (CPIC00342). D, *P. varians* from the Hawaiian Islands (CPIC00357). E, *P. varians* from the Hawaiian Islands (CPIC00358). F, *P. varians* from the Hawaiian Islands (CPIC00405). G, *P. varians* from the Hawaiian Islands (CPIC00409). H, *P. weberi* juvenile from Luzon, Philippines (CASIZ177651). I, *P. weberi* from Luzon, Philippines (CASIZ181282). J, *P. weberi*, original description illustration from Indonesia (Bergh, 1905).

Figure 15. Scanning electron micrographs of the radula and jaws of *Pleurobranchus varians* from the Hawaiian Islands (CPIC00351). A, innermost lateral teeth, scale bar = 50 μm. B, mid-lateral teeth, scale bar = 50 μm. C, outermost lateral teeth, scale bar = 100 μm. D, jaw elements, scale bar = 100 μm.

Figure 16. Scanning electron micrographs of the radula and jaws of *Pleurobranchus varians* from the Hawaiian Islands (CPIC00358). A, innermost lateral te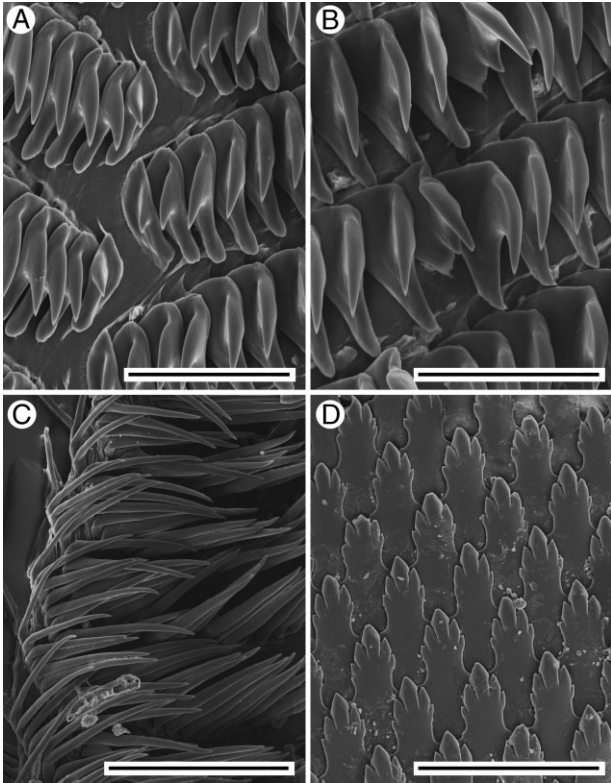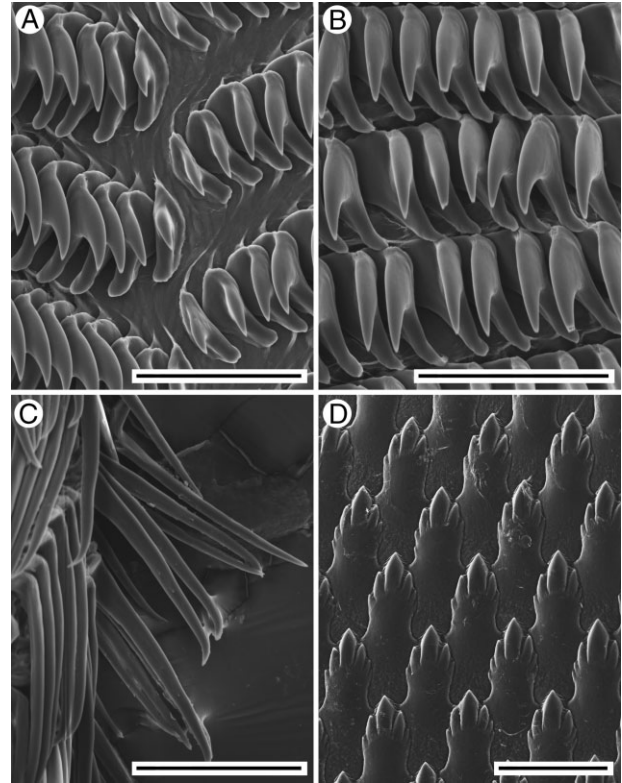eth, scale bar = 50 μm. B, mid-lateral teeth, scale bar = 50 μm. C, outermost lateral teeth, scale bar = 50 μm. D, jaw elements, scale bar = 50 μm.

External morphology and coloration: Body size up to 70 mm long. Rhinophores with multiple horizontal striations along the length. Foot does not project from the mantle. Gill rachis tuberculate at the base of the pinnae. Tubercles polygonal in shape and larger, more flattened than those in *P. albiguttatus*. Tubercles similar in size in some specimens, whereas in others tubercles become smaller towards the edge of the mantle.

Background colour orange to dark red. Opaque white pigment on some of the tubercles in some specimens, similar to *P. albiguttatus*. Oral veil and rhinophores same colour as the mantle (Fig. 14A–G).

Internal anatomy: Shell oval in shape and the posterior end is narrower than the anterior end. Protoconch roughly 300 μm, has about one whorl and lacks any ornamentation (Fig. 3A, D).

Elongated cruciform elements on jaws have a prominent cusp flanked by two to three short denticles on each side (Figs 15D, 16D). Radular formula 38 × 65.0.65 (CPIC 00351), 51 × 72.0.72 (CPIC 00357), and 51 × 58.0.58 (CPIC00358). Radular teeth smooth and hook-shaped and the inner lateral teeth are the shortest. Mid-lateral teeth 1–2× the length of the inner lateral teeth, and outermost lateral teeth 5–6× the length of the mid lateral teeth. (Figs 15A–C, 16A–C).

Reproductive system (Fig. 17) diaulic. Ampulla simple, thick, muscular duct splitting into two ducts, one connecting to the bursa copulatrix and the other to the prostate. No connection with the female gland complex was observed, but this could be because of the small size of the reproductive system. Bursa copulatrix large and rounded connecting to two other ducts, one opening into the vagina and the other to the short, somewhat rounded and irregular seminal receptacle. Vaginal duct straight and simple. Prostate large, oval and connected by a short duct to the large, irregular penis.

Remarks: The name *P. varians* was originally introduced by Pease (1860) for an animal collected in the Hawaiian Islands. Kay (1979) considered *P. varians* to be unidentifiable, and since then, this name has not been used as valid in the literature. Our phylogenetic analyses show that a subset of animals from the Central Pacific (Fig. 1), but mainly from the Hawaiian Islands,
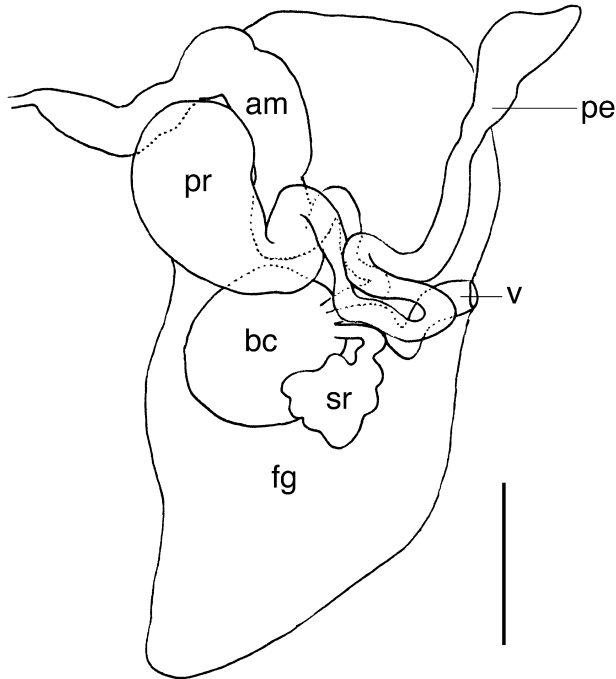
Figure 17. Reproductive system of *Pleurobranchus varians* from the Hawaiian Islands (CPIC00357), scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; fg, female gland complex; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.

are genetically distinct from the other species of *Pleurobranchus* included in the analysis. These specimens externally match the original description of *P. varians*; therefore, we reinstate the species name *P. varians* for this subset of specimens as they are genetically distinct from other species of *Pleurobranchus*.

Members of this species have recently been incorrectly identified as *P. albiguttatus* (Gosliner *et al*., 2008), a similar-looking species from the Indo-Pacific, leading to the decline in the use of the name *P. varians*. *Pleurobranchus varians* differs from *P. albiguttatus* by its larger, more polygonal-shaped tubercles. Additionally, the white pigment found on some specimens of *P. varians* is far more prevalent than on specimens of *P. albiguttatus*. *Pleurobranchus albiguttatus* seems to have a broad range of colour forms, whereas *P. varians* has a sharp distinction between only two colour forms, one with a large coverage of white pigment, one with none whatsoever.

PLEUROBRANCHUS AREOLATUS MÖRCH, 1863
(FIGS 18A–G, 19C–F, 20–22)

Pleurobranchus areolatus Mörch, 1863: 28, 29 (type locality: St Thomas, US Virgin Islands)
    Pleurobranchus atlanticus Abbott, 1949: 73–78, pl. 5, figs 1–10 (type locality: Biscayne Bay, Florida, USA)

Pleurobranchus crossei Vayssière, 1896b: 353–354, fig. 1 (type locality: Caribbean Sea) **syn. nov.**
    Pleurobranchus evelinae Thompson, 1977: 108–110, figs 12E, F, 13C–E (type locality: St. Ann's Bay, Jamaica) **syn. nov.**
    Susania gardineri White, 1952: 106, 107: pl. 6, fig. 1 (type locality: Dry Tortugas, Florida, USA)
    Pleurobranchus reesi White, 1952: 107–109: pl. 6, fig. 2 (type locality: Dry Tortugas, Florida, USA) **syn. nov.**
    Pleurobranchus emys Ev. Marcus, 1984: 70, figs 62–66 (type locality: Santa Marta, Colombia) **syn. nov.**

Type material: Type material of *P. areolatus* is not known to exist, not found at ZMUC. Holotype of *P. atlanticus* (USNM 574352), four paratypes (USNM 574342), one paratype shell (ANSP 184350), and another paratype shell (MCZ 165951). Holotype of *P. crossei* in MNHN. Holotype of *P. evelinae* (BMNH 19773W). Types of *P. gardineri* and *P. reesi* are not known to exist, not found at BMNH. No type material examined.

Material examined: Bahamas (LACM173235); Bahamas (LACM173236); Conch Point, Colón Island, Panama (LACM2004-12.1); Bocas del Toro, Panama (voucher number unknown); Panama, Caribbean Sea (Box4.No.60PanCar); Costa Rica (MZUCR-INB0001495902); Limón, Costa Rica (MZUCR-INB0003758925).

Distribution: Known from Venezuela, Jamaica, St Thomas, Aruba, St Maarten/St Martin, Brazil, Panama, México, Costa Rica, Bahamas, Puerto Rico, and Bermuda (Valdés *et al*., 2006).

External morphology and coloration: Body size up to 150 mm long (Valdés *et al*., 2006). Rhinophores with horizontal striations from the base to the tip. Posterior end of the foot projects from the mantle in some specimens. Gill rachis located at the base of the pinnae and axes of the pinnae tuberculate. Tubercles polygonal in shape. Tubercles similar in size in some specimens, in others they are smaller in size towards the edge of the mantle. Pedal gland visible in the posterior region of the pedal sole in some specimens.

Background colour light brown to deep violet, some animals with dark outlines. Opaque white pigment on some tubercles in some specimens, often arranged in a symmetrical pattern anterior to posterior, in other specimens no white pigment visible. Oral veil and rhinophores same colour as the mantle, sometimes with opaque white spots. Foot semitransparent and yellowish-brown with greyish-brown irregular spots (Fig. 18A–G).

Internal anatomy: Shell oval in shape and the posterior end is narrower than the anterior end. Protoconch roughly 400 μm, has about one whorl, and lacks any ornamentation (Fig. 19C–F).
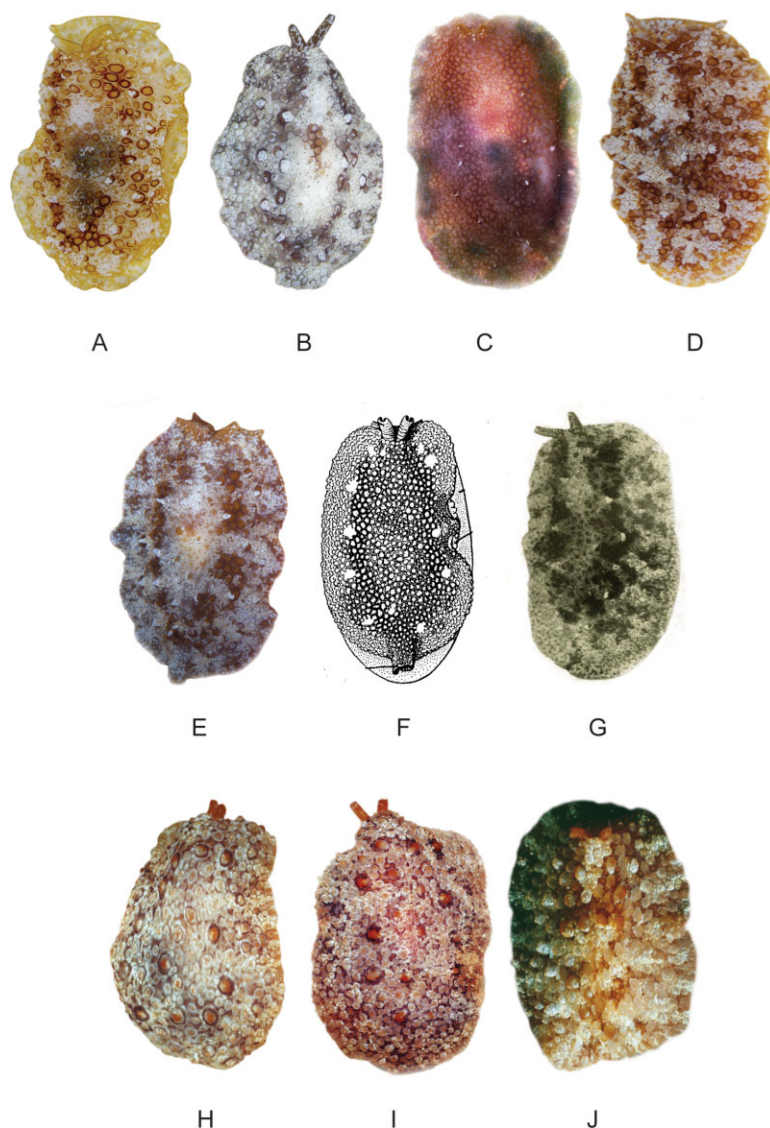
Figure 18. Colour variation in *Pleurobranchus areolatus* and *Pleurobranchus digueti*. A, *P. areolatus* from Bahamas (LACM173235). B, *P. areolatus* from Bahamas (LACM173236). C, *P. areolatus* juvenile from Colón Island, Panama (LACM2004-12.1). D, *P. areolatus* from Yucatán, México (CPIC00208). E, *P. areolatus* from Panama (CPIC00929). F, *Pleurobranchus evelinae*, original description illustration from Jamaica (Thompson, 1977). G, *Pleurobranchus atlanticus*, original description illustration from Florida (Abbott, 1949). H, *P. digueti* from Panama (MZUCR6986). I, *P. digueti* from Panama (MZUCR6201). J, *P. digueti* from Baja California, México (LACMA.9555).

Elongated cruciform elements on jaws have a prominent cusp flanked by one to two short denticles on each side (Figs 20D, 21D). Radular formula 73 × 115.0.115 (CPIC 00929). Radular teeth smooth and hook-shaped and the inner lateral teeth are the shortest. Mid-lateral teeth are 2× the length of the innermost and outermost lateral teeth roughly 5× the length of the mid-lateral teeth. (Figs 20A–C, 21A–C).

Reproductive system (Fig. 22A–B) triaulic. Ampulla convoluted, muscular duct splitting into two ducts, one connecting to the bursa copulatrix and the other to the prostate and the female gland complex. Bursa copulatrix large and rounded with two other ducts connecting to it, one opens to the vagina and the other to the short, somewhat rounded and irregular seminal receptacle. Vaginal duct straight and simple. Prostate long, simple, and connected by a short duct to the large and variable penis.

Remarks: Mörch (1863) described *P. areolatus* from the island of St Thomas in the Caribbean Sea. It has subsequently been considered a valid species by
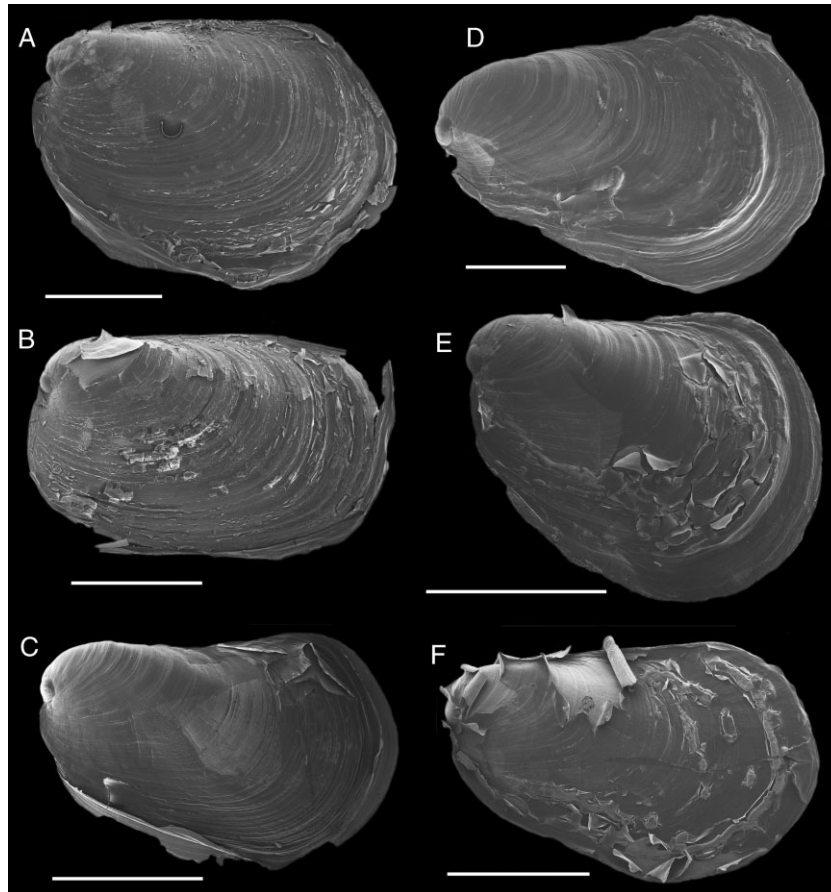
Figure 19. Scanning electron micrographs of shells. A, *Pleurobranchus digueti* from Baja California, México (LACMA.9555), scale bar = 2 mm. B, *P. digueti* from Baja California, México (LACM66-25), scale bar = 2 mm. C, *Pleurobranchus areolatus* from Colón Island, Panama (LACM2004-12.1), scale bar = 2 mm. D, *P. areolatus* from Bahamas (LACM173236), scale bar = 1 mm. E, *P. areolatus* from Costa Rica (MZUCR-INB0001495902), scale bar = 2 mm, F, *P. areolatus* from Limón, Costa Rica (MZUCR-INB0003758925), scale bar = 2 mm.

Pilsbry (1895), Bergh (1905), Marcus & Marcus (1962), and Thompson (1977). Several other species of *Pleurobranchus* have been introduced for Caribbean specimens (see list of synonyms), based on external colour differences. In the present study we sequenced several specimens covering the colour range of Caribbean *Pleurobranchus* (Fig. 18A–E) and concluded they were all the same species.

Although the type material of *P. areolatus* is lost, Bergh (1897: 111–113, pl. 9, figs 31–41) dissected and illustrated two of Mörch's type specimens. The illustrations of the penis, radula, jaw, and shell are very similar to those of the specimens here assigned to *P. areolatus*, confirming that our specimens are correctly identified.

Marcus & Marcus (1967) extended the range of *P. areolatus* into Baja California in the eastern Pacific and Edmunds (1968) reported *P. areolatus* from the western coast of Africa. The specimen identified by Edmunds (1968) as *P. areolatus* was later re-examined

and identified as *P. reticulatus* by Cervera *et al*. (1996), a species described from that region. Bertsch & Smith (1973) argued that the range extension proposed by Marcus & Marcus (1967) implied that *P. digueti*, a species described from the eastern Pacific, should be considered a synonym of *P. areolatus*. They found anatomical similarities between these two species that they provided as evidence of this synonymy.

The molecular data do not support the synonymy between *P. digueti* and *P. areolatus* (Fig. 1). Additionally, further investigation into morphological characteristics has provided evidence that supports the molecular data in separating these two species. Externally, these two species have a similar coloration that ranges from light brown to dark red. Both *P. areolatus* and *P. digueti* have opaque white pigment present on the mantle, although in *P. areolatus* the abundance and concentration of this white pigment seem to be much more variable. The morphology of the tubercles is also different. In *P. areolatus* the
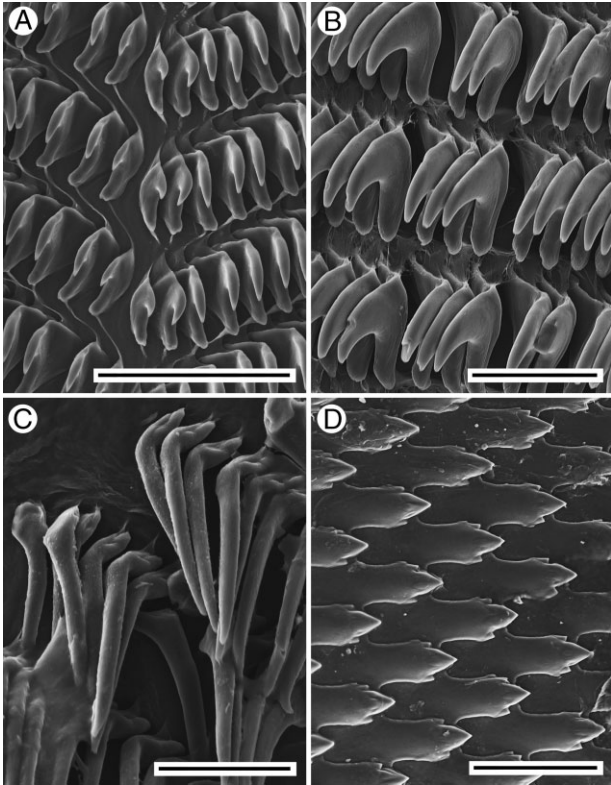
Figure 20. Scanning electron micrographs of the radula and jaws of *Pleurobranchus areolatus* from Colón Island, Panama (LACM2004-12.1). A, innermost lateral teeth, scale bar = 100 µm. B, mid-lateral teeth, scale bar = 50 µm. C, outermost lateral teeth, scale bar = 50 µm. D, jaw elements, scale bar = 100 µm.
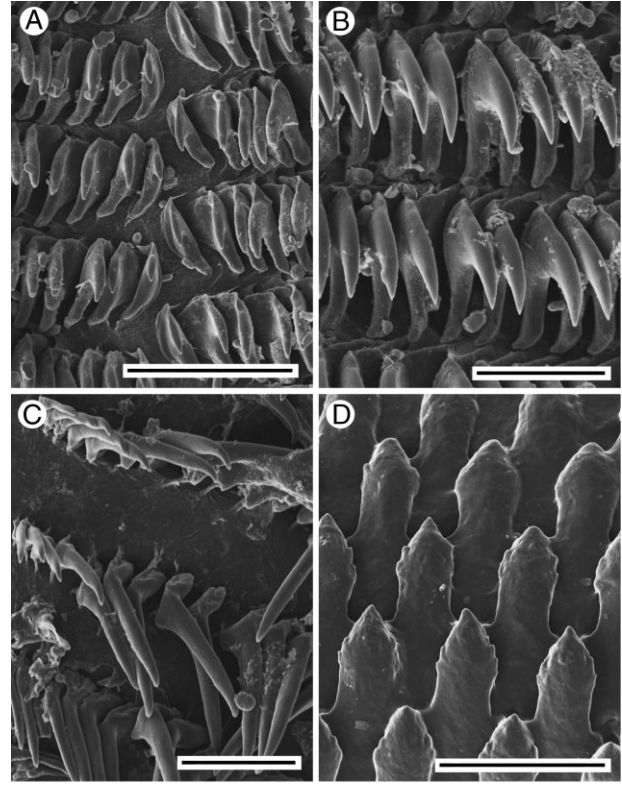

Figure 21. Scanning electron micrographs of the radula and jaws of *Pleurobranchus areolatus* from the Bahamas (LACM173236). A, innermost lateral teeth, scale bar = 100 µm. B, mid-lateral teeth, scale bar = 50 µm. C, outermost lateral teeth, scale bar = 50 µm. D, jaw elements, scale bar = 100 µm.

tubercles are polygonal, relatively flat, and fairly regular, and in some individuals the tubercles decrease in size towards the edge of the mantle. Conversely, individuals of *P. digueti* have very large, spike-shaped tubercles that cover part of the mantle and are surrounded by small, polygonal tubercles such as we see in *P. areolatus*. The rhinophores are similar in these two species, both having bands of superficial opaque white pigment present, but these are more conspicuous in *P. areolatus*.

There are also numerous differences in internal morphology. Whereas both *P. areolatus* and *P. digueti* have similar radular formulas and smooth, hook-shaped radular teeth (Figs 20, 21, 23, 24), the radulae of *P. areolatus* are much shorter than those of *P. digueti*. The jaws of *P. digueti* generally have more denticles on the elongated cruciform elements than individuals of *P. areolatus* (Figs 20D, 21D, 23D, 24D). The shells of these two species also differ. Although both species have oval-shaped shells, in *P. areolatus* the posterior region of the shell is much narrower than the anterior portion (Fig. 19C–F), whereas in *P. digueti* the width is uniform

throughout the length of the shell (Fig. 19A, B). Finally, the reproductive system of these two species is substantially different. Both the ampulla and the prostate are much longer and thinner in *P. digueti*, as is the deferent duct (Fig. 25). The seminal receptacle is also much larger in *P. digueti* (Fig. 25) and far more irregular than in *P. areolatus* (Fig. 22). Lastly, the bursa copulatrix seems to be smaller in *P. digueti*, and the duct connecting it to the vagina much wider (Fig. 25).

As mentioned above, the Brazilian morphotype generally identified as *P. areolatus* (Fig. 7A–B) represents, in fact, *P. reticulatus*. However, the presence of *P. areolatus* in Brazil is confirmed based on the specimen illustrated by Padula *et al.* (2012) as *P. atlanticus*.

PLEUROBRANCHUS GRANDIS PEASE, 1868
(FIG. 12E, F)

Pleurobranchus grandis Pease, 1868: 78, 79, pl. 10, fig. 2 (type locality: Huahine, French Polynesia)

?*Pleurobranchus blainvillii* Lesson, 1830: 143, pl. 51, fig. 1 (type locality: Point Venus, Tahiti)
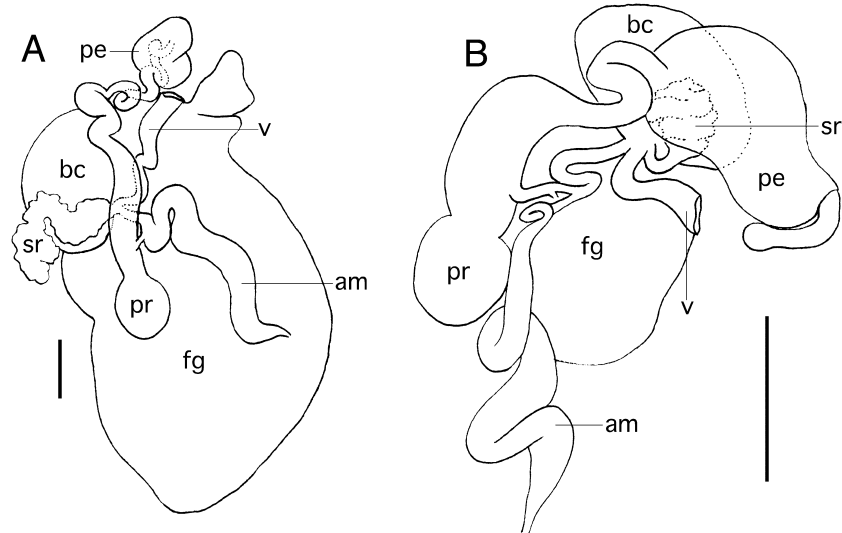
Figure 22. Reproductive system of *Pleurobranchus areolatus*. A, specimen from Limón, Costa Rica (MZUCR-INB0003758925); B, specimen from Colón Island, Panama (LACM2004-12.1), scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; fg, female gland complex; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.
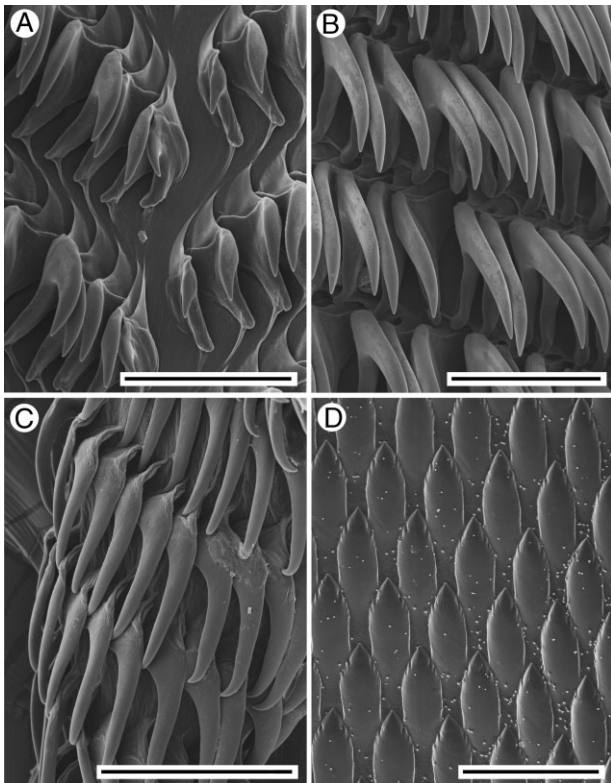
Figure 23. Scanning electron micrographs of the radula and jaws of *Pleurobranchus digueti* from Baja California, México (LACMA.9555). A, innermost lateral teeth, scale bar = 100 µm. B, mid-lateral teeth, scale bar = 100 µm. C, outermost lateral teeth, scale bar = 100 µm. D, jaw elements, scale bar = 200 µm.
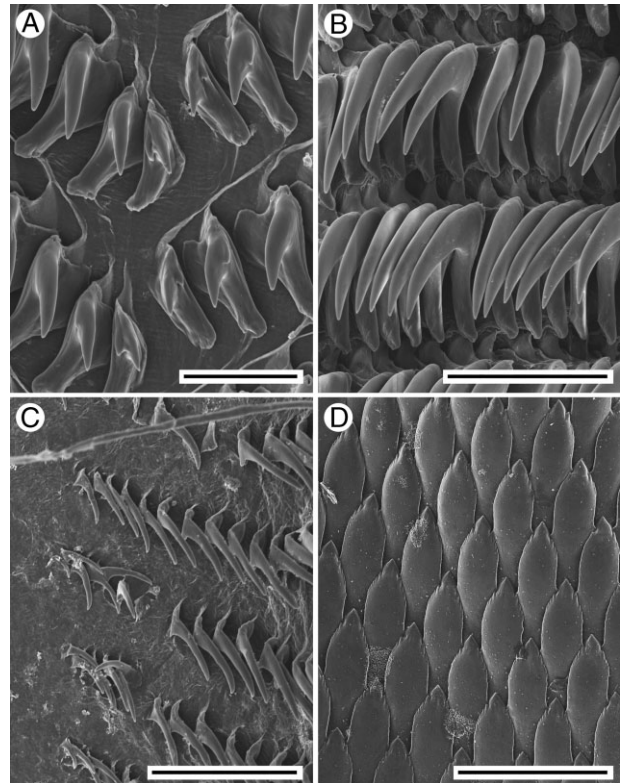
Figure 24. Scanning electron micrographs of the radula and jaws of *Pleurobranchus digueti* from Baja California, México (LACM66-25). A, innermost lateral teeth, scale bar = 50 µm. B, mid-lateral teeth, scale bar = 100 µm. C, outermost lateral teeth, scale bar = 100 µm. D, jaw elements, scale bar = 200 µm.
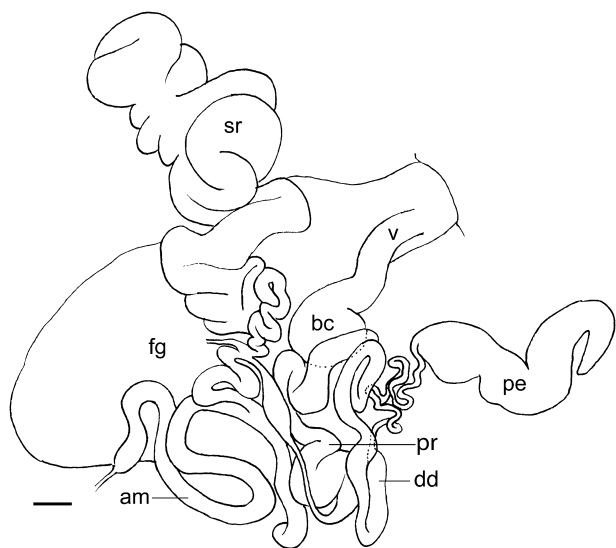
Figure 25. Reproductive system of *Pleurobranchus digueti* from Baja California, México (LACMA.9555), scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland complex; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.

?*Pleurobranchus violaceus* Pease, 1863: 510 (type locality: 'Sandwich Islands' [Hawaiian Islands])

Type material: Type material of *P. grandis* is not known to exist, not found at BMNH or BPBM. Types of *P. blainvillii* are not known to exist, not found at MNHN (Valdés & Héros, 1998). Types of *P. violaceus* are not known to exist, not found at BMNH or BPBM.

Material examined: Juvenile from New Caledonia (no voucher no.).

Distribution: Known from New Guinea, the Philippines, Fiji, Society Islands, and New Caledonia (Gosliner *et al.*, 2008).

External morphology and coloration: Description provided in Er. Marcus & Ev. Marcus (1970).

Internal anatomy: Description provided in Er. Marcus & Ev. Marcus (1970) and Martynov & Schrödl (2009).

Remarks: Pleurobranchus grandis was originally described and illustrated by Pease (1868) and has been recognized as a valid species by Pilsbry (1895) and Bergh (1897).

   Based on the original descriptions, two other species names, *P. blainvillii* Lesson, 1830 and *P. violaceus* Pease, 1863, may be synonymous with *P. grandis*. The colour patterns of these two species were both described as having a violet-coloured mantle with bluish-white striae (Lesson, 1830) or darker purple granules that give the mantle a reticulated appearance (Pease, 1860). Both could potentially be descriptions of the juvenile form

of *P. grandis*, which has a translucent, light purple body with white reticulated lines and dark purple tubercles on the mantle. Given that the descriptions are not exact matches and no dimensions of the animals were given, we are unable to determine their identity with certainty. Additionally, adults of *P. grandis* tend to have tubercles arranged in clusters and have varying proportions of red, yellow, white, and black pigment on the mantle (Fig. 12E, F). Neither of the two descriptions is similar to a mature *P. grandis*. If these three species were found definitively to be synonymous, *P. grandis* and *P. violaceus* would have to be synonymized with *P. blainvillii*, as it is the senior name. However, the descriptions of these two species are not complete enough for us to confidently recognize this synonymy; therefore, *P. grandis* is considered a valid species for the purposes of this paper.

PLEUROBRANCHUS HILLI (HEDLEY, 1894) (FIG. 12H)

Oscanius hilli Hedley, 1894: 126–128, pl. 7 (type locality: Broken Bay, New South Wales, Australia)

Type material: Holotype (AM C.645.001) and three paratypes (AM C.240097.001).

Distribution: Known from south-eastern Australia from central New South Wales to Tasmania, Victoria, and South Australia (Rudman, 2003).

External morphology and coloration: Description provided in Thompson (1970).

Internal anatomy: Description provided in Thompson (1970).

Remarks: The original description and illustration of this species was published by Hedley (1894) as *Oscanius hilli* from New South Wales, Australia, and this species has been recognized as valid by Pilsbry (1895), Bergh (1897), Thompson (1970), and Burn (2006).

   In addition to the unique range of this species compared with other members of *Pleurobranchus*, *P. hilli* is distinguishable externally from other species in this group by the large, warty, irregular tubercles on the mantle (Fig. 12H). No other described *Pleurobranchus* occurring in this region match the description of *P. hilli*.

PLEUROBRANCHUS DIGUETI ROCHEBRUNE, 1895
(FIGS 18H–J, 19A, B, 23–25)

Pleurobranchus digueti Rochebrune, 1895: 240 (type locality: Mogote, Bahía de La Paz, México)

Type material: Five syntypes known to exist (MNHN; BPBM 258974). No type material examined.

Material examined: Panama, Pacific Ocean (MZUCR 6986, MZUCR 6201); La Paz, Baja California Sur,

México (LACM A.9555); Ceralvo Island, Baja California, México (LACM 66-25).

Distribution: Known from Santa Barbara, California, USA, Gulf of California, México, to Colombia and Islas Galápagos, Ecuador (Behrens & Hermosillo, 2005).

External morphology: Body size up to 106 mm long. Rhinophores with horizontal striations from the base to the tips. Posterior end of the foot projecting from the mantle in some specimens. Gill rachis tuberculate at the base of the pinnae. Tubercles large, tentacular-shaped with pointed apices, surrounded by smaller, polygonal tubercles. Tubercles smaller towards the edge of the mantle in some specimens. Pedal gland visible in the posterior region of the pedal sole.

Background colour light brown, with opaque white pigment on some tubercles. Oral veil and rhinophores same colour as the mantle, sometimes with opaque white spots. Foot semitransparent and yellowish brown with greyish-brown, irregular spots (Fig. 18H–J).

Internal anatomy: Shell oval in shape and the same width along its length. Protoconch roughly 250 µm, has about one whorl and lacks any ornamentation (Fig. 19A–B).

Elongated cruciform elements on jaws have a prominent cusp flanked by one to two short denticles on each side (Figs 23D, 24D). Radular formula 70 × 123.0.123 (LACM A.9555), 36 × 67.0.67 (LACM 66-25), and 64 × 119.0.119 (LACM 70-1). Radular teeth smooth and hook-shaped and the innermost lateral teeth are the shortest. Mid-lateral teeth are 5× the length of the inner teeth and the outermost lateral teeth are only slightly longer than the mid-lateral teeth (Figs 23A–C, 24A–C).

Reproductive system (Fig. 25) triaulic. Ampulla long, thin, convoluted muscular duct splitting into three ducts, one connecting to the female gland complex, one to the seminal receptacle and the other to the prostate. Seminal receptacle large, elongate, and irregular. Prostate long, thin, and convoluted narrowing into a long and convoluted deferent duct. Deferent duct connects to a large and elongated penis. Bursa copulatrix large and oval and connects directly to the vagina.

Remarks: Pleurobranchus digueti was described by Rochebrune (1895) from a specimen collected by Diguet in Mogote, Bahía de la Paz, Baja California del Sur, México, also in 1895. Pilsbry (1895) redescribed *P. digueti* based on an additional specimen, collected by W. K. Fisher from La Paz. MacFarland (1966) then included extensive anatomical studies under the name *P. digueti*. The molecular and morphological information here presented clearly indicates that *P. digueti* is a valid and distinct species (see *P. areolatus*).

PLEUROBRANCHUS ALBIGUTTATUS (BERGH, 1905)
(FIGS 12A–D, 26B, 27, 28)

Oscaniella albiguttata Bergh, 1905: 58–60, pl. 2, fig. 3, pl. 11, figs 10–17 (type locality: Pasi Tanette, South Sulawesi, Indonesia)

Type material: Three syntypes of *P. albiguttatus* from Siboga station 213 mentioned in the original description, two of these syntypes in NL (ZMA.MOLL.139284).

Material examined: Madang, Papua New Guinea (CASIZ191076, CASIZ191141, CASIZ191409).

Distribution: Known from Tanzania, the Red Sea, Reunion, the Comores, Australia, New Caledonia, Papua New Guinea, Indonesia, the Philippines, Japan, Palau, and Guam (Gosliner *et al.*, 2008).

External morphology and coloration: Body size up to 65 mm long (Gosliner *et al.*, 2008). Rhinophores with multiple horizontal striations along the length. Foot sometimes projecting posteriorly from the mantle. Tubercles conical or hemispherical in profile and polygonal from above. Tubercles often smaller than those of *P. varians* or *P. peronii* and in some specimens decrease in size towards the edge of the mantle.
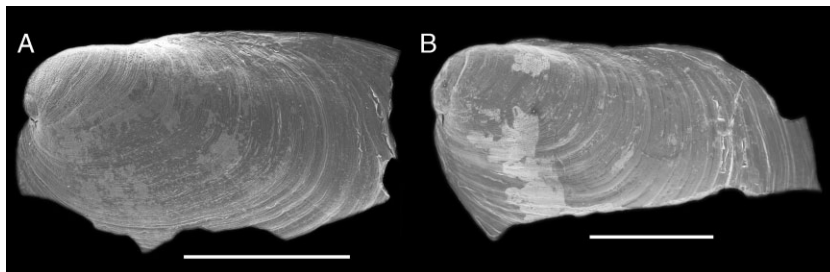

Figure 26. Scanning electron micrographs of the radula and jaws of shells. A, *Pleurobranchus weberi* from Luzon, Philippines (CASIZ181282), scale bar = 2 mm. B, *Pleurobranchus albiguttatus* from Madang, Papua New Guinea (CASIZ191076), scale bar = 1 mm.
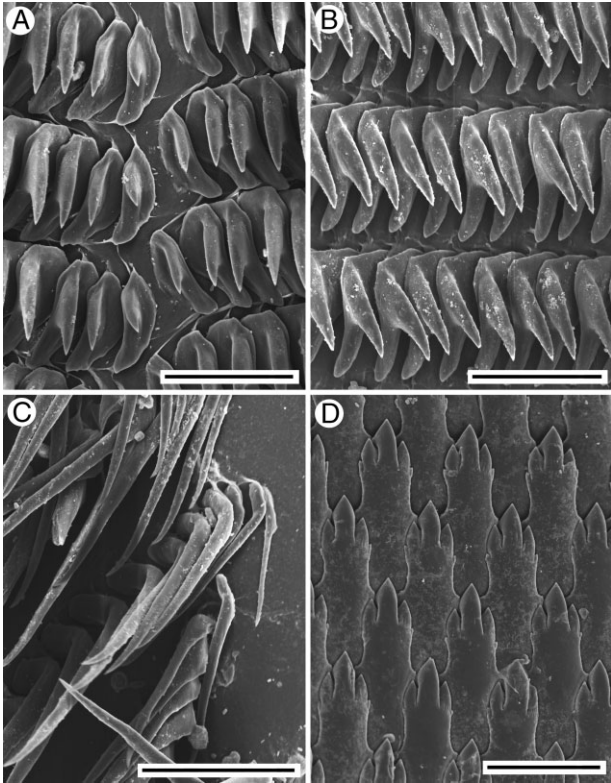
Figure 27. Scanning electron micrographs of the radula and jaws of *Pleurobranchus albiguttatus* from Madang, Papua New Guinea (CASIZ191076). A, innermost lateral teeth, scale bar = 50 μm. B, mid-lateral teeth, scale bar = 50 μm. C, outermost lateral teeth, scale bar = 50 μm. D, jaw elements, scale bar = 50 μm.
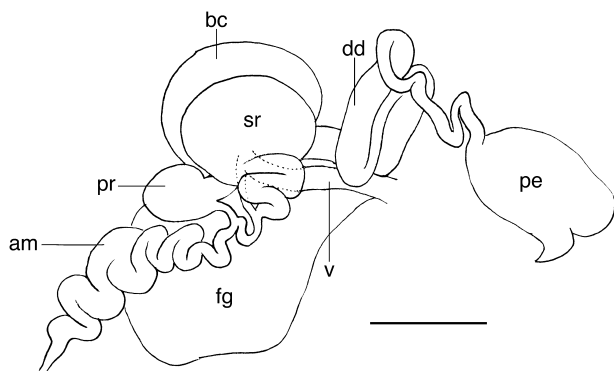

Figure 28. Reproductive system of *Pleurobranchus albiguttatus* from Madang, Papua New Guinea (CASIZ191076), scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland complex; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.

Background colour orange to dark red. White pigment sometimes present on the tubercles, and the amount of white pigment highly variable. Oral veil and rhinophores same colour as the mantle (Fig. 12A–D).

Internal anatomy: Shell is oval in shape and seems to narrow anteriorly. Protoconch is roughly 275 μm, has about one whorl, and lacks any ornamentation (Fig. 26B).

Elongated cruciform elements on jaws have a prominent cusp flanked by one large denticle on each side (Fig. 27D). Radular formula 53 × 72.0.72 (CASIZ 191076). Radular teeth smooth and hook-shaped and the innermost lateral teeth are the shortest. Mid-lateral teeth 1–2× the length of the inner lateral teeth, and outermost lateral teeth roughly 4–6× the length of the mid lateral teeth. (Fig. 27A–C).

Reproductive system (Fig. 28) diaulic. Ampulla tightly packed, convoluted, muscular duct splitting into two ducts, one connecting to the bursa copulatrix and seminal receptacle and the other to the prostate. No connection with the female gland complex was observed, but this could be because of the small size of the reproductive system. Bursa copulatrix large and rounded with two other ducts connecting to it, one opening into the vagina and the other to the large and rounded seminal receptacle. Vaginal duct straight and simple. Prostate short and rounded, connected by a long, convoluted deferent duct to the large penis.

Remarks: Bergh (1905) originally illustrated and described this species, placing it in the genus *Oscaniella* Bergh, 1897, which Thiele (1931) synonymized with *Pleurobranchus*. Later, Cervera *et al*. (1996) recognized the validity of *P. albiguttatus*.

Pleurobranchus albiguttatus is distinguishable from *P. peronii*, the most similar species morphologically to it, by its uniform conical tubercles, which often have opaque white markings covering part or the entire surface of some tubercles. In addition, the rhinophores have a uniform colour from the base to the tips.

Gosliner *et al*. (2008) listed *P. nigropunctatus* (Bergh, 1907) as a synonym of *P. albiguttatus*, although no evidence was provided. The original description of *P. nigropunctatus* states that the specimens were covered by polygon-shaped areas up to 3–5 mm in diameter, which may or may not contain a very pronounced black centre. This colour pattern is not consistent with *P. albiguttatus*. Based on this disparity in external morphology, we propose that *P. nigropunctatus* may be a valid species. However, we have no specimens from the type locality of *P. nigropunctatus*, and as such we are unable to provide definitive evidence for this claim.

Figure 29. Scanning electron micrographs of the radula and jaws of *Pleurobranchus weberi* from the Hawaiian Islands (CASIZ160491). A, innermost lateral teeth, scale bar = 100 μm. B, mid-lateral teeth, scale bar = 100 μm. C, outermost lateral teeth, scale bar = 100 μm. D, jaw elements, scale bar = 200 μm.

Figure 30. Scanning electron micrographs of the radula and jaws of *Pleurobranchus weberi* from Luzon, Philippines (CASIZ177651). A, innermost lateral teeth, scale bar = 30 μm. B, mid-lateral teeth, scale bar = 20 μm. C, outermost lateral teeth, scale bar = 20 μm. D, jaw elements, scale bar = 50 μm.

PLEUROBRANCHUS WEBERI (BERGH, 1905)
(FIGS 14H–J, 26A, 29–31)

Oscanius weberi Bergh, 1905: 53–55, pl. 2, fig. 1, pl. 6, figs 1–6 (type locality: Selayar Island, Indonesia)

   ?*Pleurobranchus tessellatus* Pease, 1861: 245 (type locality: Pacific Ocean)

Type material: Four syntypes of *Oscanius weberi* mentioned in the original description, one from station 213 (not in NL), one from station 303 in NL (ZMA.MOLL.139379), two from station 201 (not in NL).

Material examined: Makena Bay, Maui, Hawaiian Islands, USA (CASIZ160491); Luzon, Philippines (CASIZ177651, CASIZ181282).

Distribution: Known from Indonesia and the Philippines (Gosliner *et al.*, 2008) and the Hawaiian Islands (present study).

External morphology: Body size up to 200 mm long (Gosliner *et al.*, 2008). Rhinophores smooth. Mantle completely covers the foot. Gill rachis heavily tuberculate at both the base of the pinnae and along its length.

Tubercles widely spaced and conical in adults, surrounded by rings of flattened secondary tubercles. In some specimens, these rings collect sand. No secondary tubercles present in juveniles.

   Background colour in mature animals cream to orange or red; multiple colours present in some individuals. Tubercles usually with darker pigment than the rest of the mantle, surrounded by one or two rings of white pigment or sand. Juveniles translucent white with orange pigment and white reticulations on the mantle. Tubercles dark purple. Oral veil and rhinophores same colour as the mantle in adults, orange in juveniles, sometimes with purple tips (Fig. 14H–J).

Internal anatomy: Shell oval in shape and the same width along its length. Protoconch roughly 300 μm, has about one whorl, and lacks any ornamentation (Fig. 26A).

   Elongated cruciform elements on jaws have a prominent cusp flanked by one to four short denticles on each side (Figs 29D, 30D, 31D). Radular formula 42 × 60.0.60 (CASIZ 177651) and 55 × 109.0.109 (CASIZ 181282). Radular teeth smooth and hook-shaped and
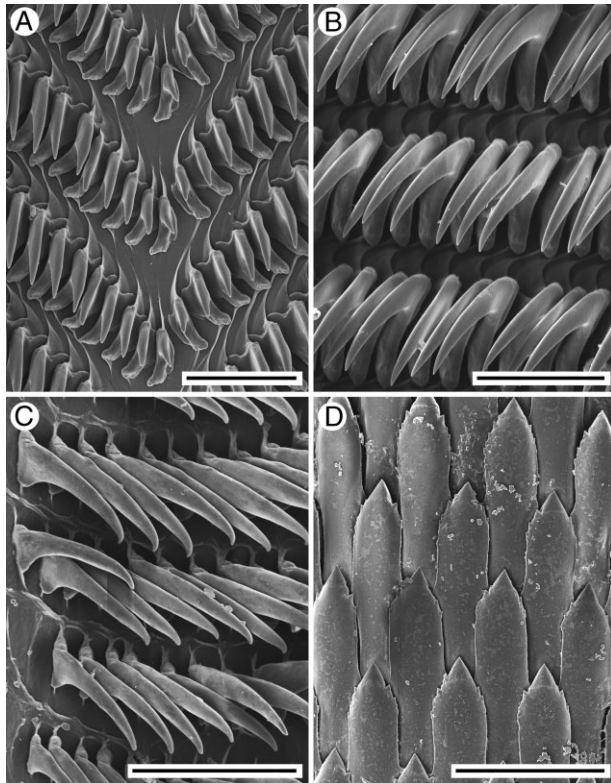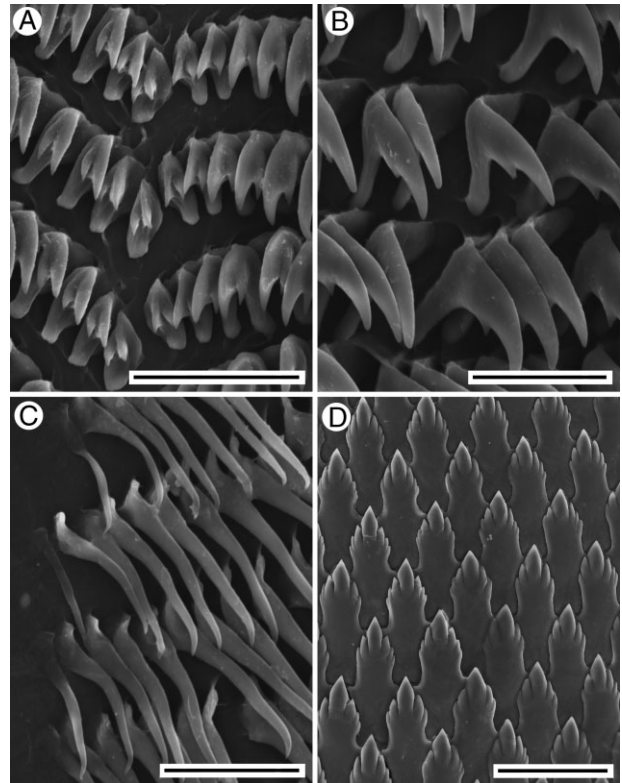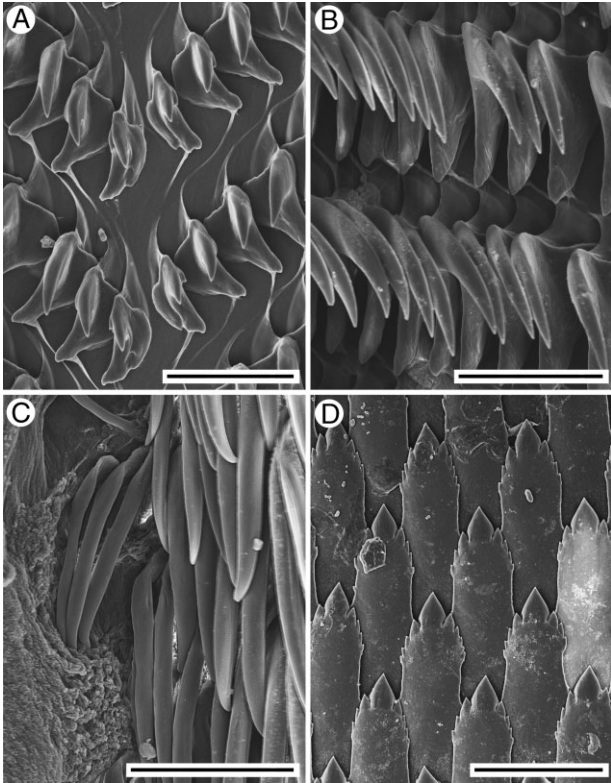
Figure 31. Scanning electron micrographs of the radula and jaws of *Pleurobranchus weberi* from Luzon, Philippines (CASIZ181282). A, innermost lateral teeth, scale bar = 50 μm. B, mid-lateral teeth, scale bar = 50 μm. C, outermost lateral teeth, scale bar = 30 μm. D, jaw elements, scale bar = 100 μm.

Figure 32. Reproductive system of *Pleurobranchus weberi* from the Hawaiian Islands (CASIZ160491), scale bar = 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland complex; pe, penis; pr, prostate; sr, seminal receptacle; v, vagina.

the innermost lateral teeth are the shortest. The mid-lateral teeth are roughly 5× the length of the inner teeth and the outermost lateral teeth are only slightly longer than the mid-lateral teeth (Figs 29A–C, 30A–C, 31A–C).

Reproductive system (Fig. 32) triaulic. Ampulla long, thin, convoluted muscular duct that splits into three ducts, one connecting to the female gland complex, one to the seminal receptacle, and the other to the deferent duct, which connects to the prostate. Seminal receptacle large and rounded. Prostate large and rounded, narrowing into a long and convoluted deferent duct that narrows further into the penis. Penis large and irregularly shaped. Bursa copulatrix large and oval, connecting directly to the vagina.

Remarks: Bergh (1905) described and illustrated *P. weberi*, originally *Oscanius weberi*, which can be distinguished from other Indo-Pacific species of *Pleurobranchus* by its very distinct dark red pigment and two opaque white concentric circles surrounding the tubercles on its mantle. Juveniles of this species are very similar to juveniles of *P. grandis*, with a trans-

lucent, light purple mantle with reticulated white lines and small, dark purple tubercles. These seem to be distinguishable from *P. grandis* by the orange pigment surrounding the tubercles and on the rhinophores.

Pleurobranchus tessellatus Pease, 1861, was considered a synonym of *P. grandis* by Rudman (2008). However, the original description indicates that this species is cream in colour with an opaque white reticulation, and irregularly maculated with reddish brown. This is consistent with the specimens here assigned to *P. weberi*. As the type material of *P. tessellatus* cannot be examined, and is not known to exist, this synonymy is regarded as tentative.

PLEUROBRANCHUS NIGROPUNCTATUS (Bergh, 1907)

Oscaniella nigropunctata Bergh, 1907: 37–39, pl. 4, figs 22–26, pl. 11, figs 9–18 (type locality: Infanta, Western Cape, South Africa)

Type material: Type material not known to exist, not found at ZMUC.

Distribution: Known only from South Africa (Gosliner, 1987).

Remarks: Originally described by Bergh (1907) as *Oscaniella nigropunctata*, *P. nigropunctatus* (Bergh, 1907), as explained above, is a potential synonym of *P. albiguttatus* or, as suggested by Rudman (1999b), a synonym of *P. peronii*. On the contrary, Gosliner (1987) considered it a valid species. The description of *P. nigropunctatus* indicates that there are darker

polygonal areas towards the edges of the mantle that supposedly have a very pronounced black centre. In addition to the absence of distinct black spots on specimens of *P. albiguttatus*, the description of *P. nigropunctatus* does not include the presence of white colour on the mantle, which is often a trait to distinguish individuals of *P. albiguttatus*. One specimen included in our molecular data is from South Africa and seems to be genetically distinct from other *Pleurobranchus*. We believe that this may be *P. nigropunctatus*, but more specimens are needed for confirmation.

DISCUSSION

TAXONOMY

The analysis of the molecular data produced well-resolved and generally well-supported phylogenetic consensus trees at the species level (Fig. 1). However, none of the analyses recovered *Pleurobranchus* as monophyletic. The most likely explanation is that the genes used in this study are not the most appropriate to resolve deep nodes within Nudipleura, but they provide valuable data to resolve the relationships amongst species. In this study, we used recently developed standards in the delimitation of problematic sea slug groups, using morphological differences, reciprocal monophyly (based on mitochondrial and nuclear gene phylogenies), and different species delimitation analyses to validate the species taxonomy (Ornelas-Gatdula *et al*., 2012; Padula, Wirtz & Schrödl, 2014b). Although most species are well supported in the phylogenetic analysis and their molecular distinctiveness is substantiated in the ABGD analyses, there are some exceptions. For example, the species *P. varians*, *P. areolatus*, and *P. albiguttatus* were grouped together in all ABGD analyses, and the monophyly of *P. varians* and *P. areolatus* is poorly supported in both Bayesian and maximum likelihood analyses of the concatenated sequences. However, *P. albiguttatus* is clearly monophyletic. The lack of support for *P. varians* and *P. areolatus* is particularly surprising, as the ranges of these two species are far apart (Central Pacific and Caribbean, respectively) and they are morphologically distinct. Because of this, they are here considered distinct valid species. Another example of problematic species includes *P. peroni*, *P. reticulatus*, and *P. garciagomezi*, which are grouped together in some ABGD analyses. In this case, the lack of enough sequence data for *P. garciagomezi* and some taxonomic uncertainties discussed above may be the cause for these results. However, *P. peroni* and *P. reticulatus* are clearly monophyletic and morphologically distinct and therefore considered as two different species.

Based on trees here obtained and a re-evaluation of the original descriptions of all species of *Pleurobranchus*, we propose a new taxonomic classification for this group, as described above and summarized in Table 5. Furthermore, after reviewing the original descriptions of all nominal species, some species previously considered members of *Pleurobranchus* were transferred to other genera and some descriptions were too incomplete for us to determine the identity of these species (Table 6).

REPRODUCTIVE ANATOMY

Willan (1987) considered the reproductive system of *Pleurobranchus* to be diaulic, whereas Cervera *et al*. (2000) regarded *Pleurobranchus* as having a modified triaulic system. Valdés, Gosliner & Ghiselin (2010) discussed the differences between the arrangement of the reproductive systems of *Pleurobranchus* and other pleurobranchid groups, which display considerable variation. In the present study we observed considerable variation within *Pleurobranchus*, with some species having a direct connection between the male and female parts of the gonoduct and lacking a uterine duct, as indicated by Cervera *et al*. (2000), and others having a true uterine duct, connecting the prostate and the bursa copulatrix to the female gland complex, as in other triaulic systems such as dorid nudibranchs (Valdés *et al*., 2010). As mentioned above, some of the specimens examined were small and the reproductive organs difficult to examine; therefore, the observations shown in this study will need further verification using histology and/or examination of large, well-preserved specimens. However, it appears that *Pleurobranchus* displays a considerable diversity of genital arrangements and could include a combination of diaulic and triaulic species as well as different types of triaulic systems, with some species having the uterine duct located on the ampula–prostate connection and others having this duct located on the male–female gonoduct connection. This diversity of arrangements may prove useful in the species-level taxonomy for this group (Martynov & Schrödl, 2009).

BIOGEOGRAPHY

The most common biogeographical structure in temperate and tropical sea slugs, as well as in many other groups of marine invertebrates, is exemplified by different groups of dorid nudibranchs, described by Gosliner & Johnson (1999), Valdés (2001), Garavoy, Valdés & Gosliner (2001), Dorgan, Valdés & Gosliner (2002), and Padula & Valdés (2012). In these groups there is a primary dichotomy in the phylogeny, with one clade containing species from the Indo-Pacific and the other containing species from the eastern Pacific and Atlantic (Fig. 33). This is consistent with the biogeographical patterns found in other marine invertebrates

Table 5. Proposed synonymies of species of *Pleurobranchus*

Species	Synonyms	Possible synonyms
Pleurobranchus peronii Cuvier, 1804	*Pleurobranchus cornutus* Quoy & Gaimard, 1832, *Pleurobranchus giardi* Vayssiere, 1896, *Pleurobranchus ovalis* Pease, 1868, *Oscaniella purpurea* Bergh, 1897, *Pleurobranchus winckworthi* White, 1946, *Pleurobranchus papillatus* Risbec, 1951, *Pleurobranchus inhacae* Macnae, 1962, *Pleurobranchus xhosa* Macnae, 1962, *Pleurobranchus hirasei* Baba, 1971	None
Pleurobranchus membranaceus Montagu, 1815	*Pleurobranchus tuberculatus* Meckel, 1808, *Lamellaria membranacea* Montagu, 1815, *Gymnotoplax barashi* Ev. Marcus, 1977	*Pleurobranchus lesuerii* Blainville, 1825, *Pleurobranchus contarinii* Vérany, 1846, *Pleurobranchus denotarisii* Vérany, 1846
Pleurobranchus forskalii Rüppell and Leuckart, 1828	'Lepus marinus' Forsskål, 1776, *Pleurobranchus perrieri* Vayssiere, 1896, *Oscanius semperi* Vayssiere, 1896	*Susania ceylonica* White, 1948
Pleurobranchus mamillatus Quoy & Gaimard, 1832	None	*Pleurobranchus moebii* Vayssiere, 1896
Pleurobranchus reticulatus Rang, 1832	None	*Pleurobranchus garciagomezi* Cervera, Cattaneo-Vietti & Edmunds, 1996
Pleurobranchus testudinarius Cantraine, 1835	None	*Pleurobranchus iouspi* Ev. Marcus, 1984
Pleurobranchus varians Pease, 1860	None	None
Pleurobranchus areolatus Mörch, 1863	*Pleurobranchus crossei* Vayssiere, 1896, *Pleurobranchus atlanticus* Abbott, 1949, *Pleurobranchus evelinae* Thompson, 1977, *Susania gardineri* White, 1952, *Pleurobranchus reesi* White, 1952, *Pleurobranchus emys* Ev. Marcus, 1984	None
Pleurobranchus grandis Pease, 1868	None	*Pleurobranchus blainvillii* Lesson, 1830 *Pleurobranchus violaceus* Pease, 1863
Pleurobranchus hilli (Hedley, 1894)	None	None
Pleurobranchus digueti Rochebrune, 1895	None	None
Pleurobranchus albiguttatus (Bergh, 1905)	None	None
Pleurobranchus weberi (Bergh, 1905)	None	*Pleurobranchus tessellatus* Pease, 1863
Pleurobranchus nigropunctatus (Bergh, 1907)	None	None

such as *Nerita*, a group of marine gastropods (Frey & Vermeij, 2008), the fiddler crab genus *Uca* (Levinton, Sturmbauer & Christy, 1996), the shrimp genus *Penaeus* (Lavery *et al.*, 2004), the sea urchin genus *Eucidaris* (Lessios, Kessing & Robertson, 1999), and multiple clades within Littorinidae (Reid, Dyal & Williams, 2010).
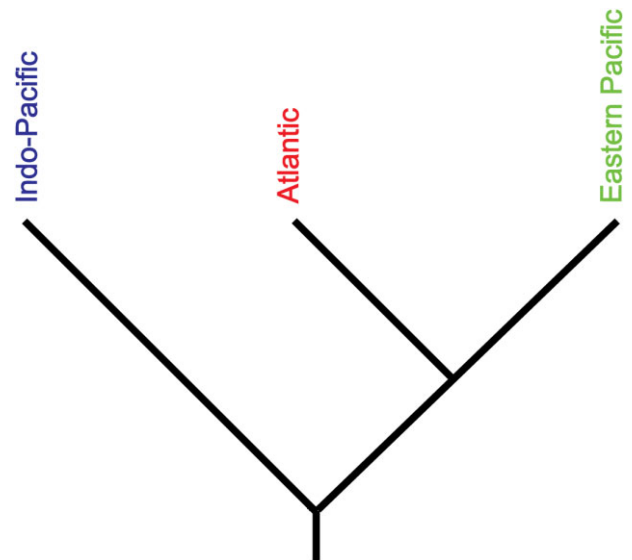
Valdés (2004) hypothesized that the pattern observed in dorid nudibranchs was determined by vicariant events related to the break-up of the Tethys Sea, including the formation of the Arabian Land Bridge 23 Mya (Harzhauser *et al.*, 2007), the East Pacific Barrier about 10 Mya (Lessios, Kessing & Pearse, 2001), and the

Table 6. Proposed classification changes to species assigned to *Pleurobranchus* and a list of the species that could not be identified

Species	New classification
Berthella or *Berthellina*	
Pleurobranchus elongatus Cantraine, 1835	*Berthella* sp.
Pleurobranchus dehaanii Cantraine, 1841	*Berthellina* sp.
Pleurobranchus brevifons Philippi, 1844	*Berthella* sp.
Pleurobranchus perforatus Philippi, 1844	*Berthella* sp.
Pleurobranchus marginatus Pease, 1860	*Berthella* sp.
Pleurobranchus rufus Pease, 1860	*Berthellina delicata* (Pease, 1861)
Pleurobranchus ornatus Cheeseman, 1878	*Berthella martensi* (Pilsbry, 1896)
Pleurobranchus monterosatoi Vayssiere, 1880	*Berthella* sp.
Pleurobranchus strubelli Bergh, 1897	*Berthella* sp.
Pleurobranchus lacteus Dall & Simpson, 1901	*Berthella* sp.
Placobranchiopsis niveus Verrill, 1901	*Berthella* sp.
Pleurobranchus caledonicus Risbec, 1928	*Berthella* sp.
Unidentified *Pleurobranchus*	*Berthella* sp.
Pleurobranchus oblongus Andouin, 1827	NA
Pleurobranchus calyptraeoides Forbes, 1843	NA
Pleurobranchus limacoides Forbes, 1843	NA
Pleurobranchus scutatus Forbes, 1843	NA
Pleurobranchus sordidus Forbes, 1844	NA
Pleurobranchus savi Vérany, 1846	NA
Oscaniella affinis Bergh, 1897	NA
Oscaniella diversicolor Bergh, 1897	NA
Oscanius petersi Bergh, 1897	NA
Oscanius semonis Bergh, 1897	NA
Oscaniella styphla Bergh, 1897	NA
Pleurobranchus lowei Watson, 1897	NA
Pleurobranchus hornelli Farran, 1905	NA
Oscaniella dubia Bergh, 1905	NA
Pleurobranchus griseus Bergh, 1905	NA
Oscaniella inermis Bergh, 1905	NA
Pleurobranchus latipes Bergh, 1905	NA
Oscaniella lugubris Bergh, 1905	NA
Oscaniella modesta Bergh, 1905	NA
Oscanius papuligerus Bergh, 1905	NA
Oscaniella purpurescens Bergh, 1905	NA
Oscanius sibogae Bergh, 1905	NA
Pleurobranchus obses White, 1946	NA

Figure 33. Geographical area cladogram for groups of dorid nudibranchs as presented by Valdés (2004).

eastern Pacific species. One hypothesis for this is that *Pleurobranchus* diversified before the break-up of the Tethys, but our molecular clock analysis estimated divergence times that are inconsistent with that premise. Some other marine invertebrate groups also break away from the common pattern, including the sea urchin group *Diadema* (Lessios *et al*., 2001), the hooded shrimps of *Betaeus* and *Betaeopsis* (Anker & Baeza, 2012), and the sea slug *Pontohedyle* (Jörger *et al*., 2012).

Our molecular clock analysis (Fig. 34, Table 4) indicates that the majority of the diversification in *Pleurobranchus* occurred in the Indo-Pacific between 1 and 10 Mya. The divergence between *Pleurobranchus* and *Berthella* is estimated to have occurred in the 10–15 Mya range and the deepest branch in *Pleurobranchus* is dated within the 8–13 Mya range. The dates for the divergence between *Pleurobranchus* and *Berthella* are significantly more modern than the estimates by Göbbeler & Klussmann-Kolb (2010), who placed the origin of *Pleurobranchus* in the Oligocene–Miocene transition. However, Göbbeler & Klussmann-Kolb's (2010) molecular clock estimates for the early diversification of *Pleurobranchus* in the middle Miocene coincide with the data presented here. The limited sampling of other Pleurobranchomorpha in the present study may account for this difference.

Considering the unique biogeographical structure of *Pleurobranchus* and the estimated divergence times for the various clades, we speculate that *Pleurobranchus* cladogenesis has been produced by a complexity of factors. For instance, a series of small vicariant events in the periphery of the Indo-Pacific region has been suggested to produce complex patterns of diversifica-

Isthmus of Panama 3.1–3.4 Mya (Coates & Obando, 1996). The implication of this hypothesis is that all the groups that follow this pattern were poorly diversified before the closure of the east–west communication in tropical and temperate oceans.

However, the phylogeny of *Pleurobranchus* differs considerably from this pattern (Fig. 34). In *Pleurobranchus* there is only one species found in the Caribbean, *P. areolatus*. Yet, contrary to the pattern mentioned above, *P. areolatus* is not sister to *P. digueti*, the only eastern Pacific species of *Pleurobranchus*. Additionally, Indo-Pacific species of *Pleurobranchus* are paraphyletic and are more closely related to the Atlantic species of *Pleurobranchus* than the extant
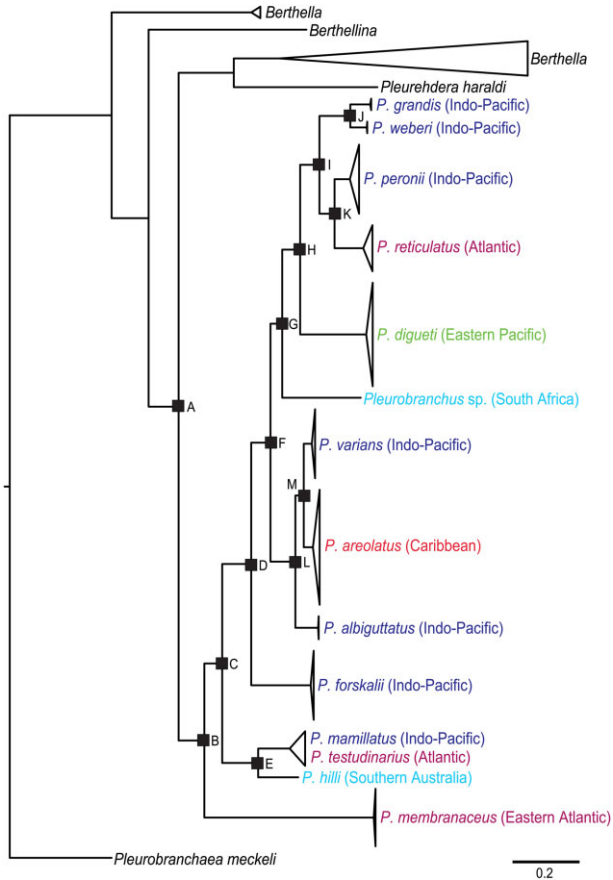
Figure 34. Simplified version of the Bayesian tree from Figure 1. Dark blue-coloured names indicate Indo-Pacific species, pink indicates eastern Atlantic species, red indicates Caribbean species, light blue indicates temperate Indo-Pacific species, and green indicates eastern Pacific species. Additionally, nodes with a black square are labelled with the proposed time of divergence from the molecular clock analysis run by r8s (Table 4) in millions of years ago (Mya).

tion in other invertebrates and sea slugs (Gosliner & Johnson, 1999; Garavoy, Valdés & Gosliner, 2001; Lessios *et al.*, 2001). A similar hypothesis could be used to explain some of divergences within *Pleurobranchus*, including the split of *P. weberi* and *P. grandis*, the split of the ancestor of the *P. weberi* and *P. grandis* clade and the ancestor of *P. peronii* and *P. reticulatus*, and the split of *P. albiguttatus* and the ancestor of *P. varians* and *P. areolatus*. These species appear to have diverged from other populations around the periphery of their original range, including those from the eastern Atlantic, eastern Pacific, and Caribbean, creating the unusual biogeographical patterns found in *Pleurobranchus*. These peripheral divergences may have been initiated by vicariant events or dispersal, but there is no definitive evidence supporting either hypothesis.

The divergence of *P. hilli* and *P. mamillatus* + *P. testudinarius* is especially interesting. The common ancestor of these species may have been isolated in the southern Indo-Pacific, then diverged into *P. hilli* and *P. mamillatus* + *P. testudinarius* as a result of a vicariant process caused by the expansion of the Sub-antarctic Front and adjacent temperate waters (Verducci *et al.*, 2009). The individuals of this hypothetical ancestor that were better adapted to colder waters evolved into *P. hilli*, a temperate species, and those that were better adapted to warmer temperatures evolved into *P. mamillatus* + *P. testudinarius*. This has also been suggested in *Nerita*, a group of primarily tropical marine gastropods (Frey & Vermeij, 2008), and the sea urchin clade *Diadema* (Lessios *et al.*, 2001).

The lack of sister species relationships between the eastern Pacific and the Caribbean species is also surprising. One possibility is that the ancestors of both *P. digueti* and *P. areolatus* had sister species that went extinct after the closure of the Isthmus of Panama, leaving the observed pattern. The dramatic temperature shifts of these two areas after the closure of the Isthmus of Panama has been suggested as a major factor in the large number of extinctions that occurred during this period (Vermeij & Petuch, 1986; Allmon *et al.*, 1993; Jackson *et al.*, 1993; Vermeij & Rosenberg, 1993; O'Dea *et al.*, 2007). Examples in the recent literature have revealed that interpretation of biogeographical signals in phylogenetic trees can be misleading because of lack of fossil data (Edwards *et al.*, 2011), such as is found in *Pleurobranchus*.

The other species in the Atlantic, *P. reticulatus* and *P. garciagomezi*, seem to have secondarily dispersed from the Indo-Pacific around 3 Mya, at the end of the Pliocene. This divergence is consistent with the strengthening of the Benguela current, which could have caused an allopatric split. This pattern is concordant with that found in species of *Diadema* (Lessios *et al.*, 2001).

SIGNIFICANCE OF COLOUR

Gosliner & Behrens (1990) estimated that 50 per cent of 'opisthobranch' molluscs are aposematic, displaying bright external colour patterns that warn predators that the slug is toxic or unpalatable. Experimental data have shown that these bright external colorations are more effective in preventing predation than distasteful cryptic species (Tullrot, 1994). However, most species of *Pleurobranchus* are nocturnal, potentially limiting the need to warn predators, and appear to have cryptic coloration and blend in while on rocks and coral rubble (Thompson & Slinn, 1959), indicating that aposematism does not seem to be a method of protection for species of *Pleurobranchus*. One species of *Pleurobranchus*, *P. garciagomezi*, is known to be active

during the day (Fontes, Tempera & Wirtz, 2001), but unlike other species of *Pleurobranchus* does not have the bright external coloration associated with aposematism.

In 'opisthobranch' molluscs, colour pattern has been used to make taxonomic decisions on species boundaries and definitions. The established paradigm in 'opisthobranch' systematics is that members of the same species share the same, or similar, colour pattern whereas members of other species have differing colour patterns. Recent molecular studies, however, have shown that in some sea slugs, colour patterns are extremely variable within the same species (Pola *et al*., 2006; Ornelas-Gatdula *et al*., 2012; Valdés, Ornelas-Gatdula & Dupont, 2013). Other studies have revealed the existence of cryptic species that form part of a species complex long thought to be one or few widespread species (Carmona *et al*., 2011, 2013, 2014; Jörger *et al*., 2012; Krug *et al*., 2013; Padula *et al*., 2014a).

In *Pleurobranchus* there seems to be both phenotypic plasticity and cryptic diversity. For example, in the Hawaiian Islands, specimens of *P. varians* were previously identified as *P. albiguttatus* (Fig. 14A, C–D, G) because the colour patterns found on individuals of these two species seem to be remarkably similar. Based on the molecular data we now know that although these two species do not seem to differ in external morphology, they do differ genetically and geographically. *Pleurobranchus varians* is found in the Central Pacific, whereas *P. albiguttatus* is found in the rest of the Indo-Pacific. Another example of difficult-to-identify *Pleurobranchus* is members of *P. forskalii* and *P. peronii*, as both have a purple colour form with small, simple tubercles that are extremely difficult to distinguish in the field (Figs 2C, 8D–F).

One example of within-species variation occurs in *P. forskalii*. Members of this species differ in colour depending on whether they are adults, which are a dark reddish-purple colour and often have a series of white semicircles on the mantle, or juveniles, which are light brown in colour and often have dark brown or black semicircles surrounding complex tubercles on the mantle. Another interesting example of within-species variation is the two colour morphs of *P. varians* and *P. areolatus*. Both species contain individuals that are either uniform in colour, normally reddish or yellow, or specimens that have tubercles variable in colour and may be partially covered in opaque, mottled white. These two species can be difficult to identify based on photographs without knowing the locality, because specimens found in the same localities have different colorations, and specimens found in the Hawaiian Islands and the Caribbean are very similar to each other. However, we found some external patterns that are consistent within some species, and may therefore be reliable in field identifications. These are discussed in the individual species descriptions in the Systematics section, and include patterns such as the distinctive white semicircular markings on dark reddish-purple adult individuals of *P. forskalii*. At this point the evolutionary significance and biological role of the colour variation amongst individuals and species within this group are unknown.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Bayesian consensus phylogenetic trees for *cytochrome c oxidase I* (*COI*), *histone 3* (*H3*), and *16S* for *Pleurobranchus*. Posterior probabilities and bootstrap values from the maximum likelihood analysis are presented for each clade above and below each branch, respectively. Robustly supported monophyletic groups are marked with an asterisk (*).
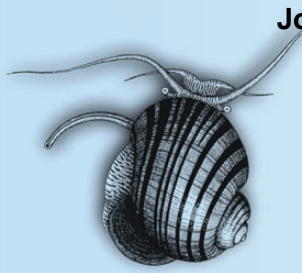
Appendix S2. 16S rDNA sequences shorter than 200 bp, not accepted by GenBank.

3. RESULTS

Chapter 10

Ortigosa D, Pola M, Carmona L, **Padula V**, Schrödl M, Cervera JL (2014) Redescription of *Felimida elegantula* (Philippi, 1844) and a preliminary phylogeny of the European species of *Felimida* (Chromodorididae). *Journal of Molluscan Studies* **80**: 541-550.

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REDESCRIPTION OF *FELIMIDA ELEGANTULA* (PHILIPPI, 1844) AND A PRELIMINARY PHYLOGENY OF THE EUROPEAN SPECIES OF *FELIMIDA* (CHROMODORIDIDAE)

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ABSTRACT

Felimida elegantula (Philippi, 1844) was originally described as *Doris elegantula* based on the external morphology of a single specimen from Sicily (Italy). Since Philippi's description, this species has been recorded only a few times, always from the Mediterranean Sea, and without any detailed description of the internal morphology. According to a recent reassessment of the family Chromodorididae, all the eastern Pacific, Atlantic and Mediterranean species previously attributed to the genus *Chromodoris* Alder & Hancock, 1855 should be reallocated to *Felimida* Marcus, 1971. Here we present a morphological redescription of *F. elegantula* based on five specimens from Sardinia (Italy) as well as a molecular phylogeny using two mitochondrial (cytochrome *c* oxidase subunit I) and16S rRNA) and one nuclear (histone-3) marker. We aim to investigate phylogenetic relationships within '*Felimida*' from the Atlantic coast of Europe and the Mediterranean Sea.

INTRODUCTION

Doris elegantula Philippi, 1844 is a poorly known species and its generic placement and phylogenetic relationships have been controversial. It was originally described based on a single specimen from Sicily (Italy) and Philippi (1844) provided information only on its external morphology. In 1880, von Ihering identified one specimen, also from Italy, as *D. elegantula*, but Pruvot-Fol (1932) stated that this was a misidentification of *Diaphorodoris luteocincta* (Sars, 1870). In addition, Pruvot-Fol (1932) described the external morphology of a single specimen of *D. elegantula* from Villefranche-sur-Mer (France) but assigned it to the genus *Glossodoris* Ehrenberg, 1831. Although she described the buccal armature and radula of the specimen, no illustrations were supplied. Sordi (1970) collected one specimen of *G. elegantula* from the Ligurian Sea (Italy) and briefly described its buccal armature, radula, penis and the egg mass. Cattaneo-Vietti & Barletta (1984) transferred the species to *Chromodoris* Alder & Hancock, 1855 and later Cattaneo-Vietti,

Chemello & Giannuzzi-Savelli (1990: pl. 3, fig. 1) gave a colour photograph of the specimen studied by Sordi (1970). Perrone (1993) found several specimens in Malta and, based on the occurrence of two distinct colour forms, proposed two subspecies: *C. elegantula elegantula*, with small dorsal red spots, and *C. elegantula polychroma*, with large dorsal spots. In addition, Perrone (1993) provided details and drawings of the radula, buccal armature and reproductive system. Later, Trainito (2003, 2005) published photographs of specimens from southern Sardinia (Italy). The most recent publication on *C. elegantula* is the record of one specimen from Turkey (Türkmen & Demirsoy, 2009).

A recent reassessment of the family Chromodorididae, based on molecular data, led Johnson & Gosliner (2012) to propose a new classification, with 14 valid genera. Among these, *Felimida* Ev. Marcus 1971 and *Dorisprismatica* d'Orbigny, 1839 were provisionally re-erected. *Felimida* was originally defined on the basis of the denticulation of the radular teeth and was said to be the only chromodorid genus bearing denticles on the inner side of the 1st to 4th innermost lateral teeth (Marcus, 1971: fig. 3).

However, Bertsch (1977) synonymized *Felimida* with *Chromodoris*, considering the differences in radular denticulation as a variation within *Chromodoris*. Johnson & Gosliner (2012), in a phylogenetic study based on two mitochondrial markers, proposed the use of the name *Felimida* for the eastern Pacific, Atlantic and Mediterranean species previously included in the genera *Chromodoris* and *Glossodoris*. However, the sampling of Atlantic chromodorids in that study was quite incomplete.

In the present contribution we focus on *Felimida elegantula*, providing a morphological redescription of the species. To test its generic placement and assess its phylogenetic relationships we have used two mitochondrial genes, cytochrome *c* oxidase subunit I (COI) and 16S rRNA (16S), and one nuclear gene, histone-3 (H3), and have analysed the species with other externally similar species allocated to *Felimida* from the Atlantic coast of Europe and the Mediterranean Sea, and the type species of the genus from the eastern Pacific Ocean. We aim to improve understanding of the relationships within *Felimida* (*sensu* Johnson & Gosliner, 2012), with the inclusion of more species, more sequences and a nuclear marker (H3).

MATERIAL AND METHODS

Morphological studies

Six specimens of *Felimida elegantula* were obtained through scuba diving in Porto San Paolo, northeastern Sardinia, Italy. One specimen was preserved in 4% formalin for histology. The remaining specimens were preserved in 96% ethanol for morphological and molecular studies. Specimens were dissected by dorsal incision. Their internal features were examined using a dissecting microscope and drawn with the aid of a camera lucida. Special attention was paid to the morphology of the reproductive system. The buccal mass was removed and dissolved in 10% sodium hydroxide until the radula and the labial cuticle were isolated from the surrounding tissue. The radula and the labial cuticle were then rinsed in water, dried and mounted for examination under a Quanta 200 scanning electron microscope.

Samples for molecular analysis

Molecular analysis included 59 specimens, representing five genera of Chromodorididae and ten *Felimida* species (Table 1). Members of six additional genera were included for comparative purposes. Voucher specimens are held at the Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN), the California Academy of Sciences, San Francisco, USA (CASIZ), Colección Nacional de Moluscos, Universidad Nacional Autónoma de México, Mexico City, México (CNMO), Museo de Zoología de la Universidad de Costa Rica, San José, Costa Rica (MZUCR), Museo de Historia Natural de El Salvador, San Salvador, El Salvador (MHNES), the Natural History Museum of Crete, Crete, Greece (NHMC), the Zoologische Staatssammlung München (ZSM), and the Zoological Museum of Bergen, Bergen, Norway (ZMBN). We obtained 34 new sequences for both COI and for H3, and 35 for 16S. Sixty-seven additional sequences from 32 specimens were obtained from GenBank (27 for COI, 30 for 16S, and 10 for H3), with emphasis on specimens and sequences used by Johnson & Gosliner (2012). *Tritonia challengeriana* Bergh, 1884 was chosen as outgroup. The classification used in this study is based on Johnson & Gosliner (2012) and Carmona *et al*. (2013) (Table 1).

DNA extraction, amplification and sequencing

DNA extractions and PCR amplifications were performed at the Universidad de Cádiz (UCA), Spain and in the Zoologische Staatssammlung München (ZSM), Germany. DNA was extracted from foot tissue of specimens preserved with 70–100%

ethanol, and performed using the DNeasy Blood and Tissue Kit Qiagen at UCA and NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co.) at ZSM, following the manufacturer's instructions. Partial sequences of COI, 16S and H3 were amplified by polymerase chain reaction (PCR) using LCO1490 and HCO2198 universal primers for COI (Folmer *et al*., 1994), 16S ar-L and 16S br-H for 16S (Palumbi *et al*., 1991) and H3AD5′3′ and H3BD5′3′ for H3 (Colgan *et al*., 1998). The master mix for the PCR was prepared in the following order: nuclease-free water up to 25 μl volume reaction, 2.5 μl of Qiagen buffer, 2.5 μl of dNTP (2 mM), 5 μl of 'Q-solution' (Qiagen), 1.5–3.5 μM magnesium chloride, 1 μl of each forward and reverse primer (10 μM), 0.25 μl of DNA polymerase (250 units) and 2–3 μl of DNA. COI amplification was performed with an initial denaturation for 3 min at 94–95 °C, followed by 39–40 cycles of 30–45 s at 94 °C, 30–45 s at 46 °C (annealing temperature) and 1–2 min at 72 °C with a final extension of 5 min at 72 °C. 16S amplification was performed with an initial denaturation for 3 min at 94–95 °C, followed by 39 cycles of 39–45 s at 94 °C, 30–50 s at 45–51.5 °C (annealing temperature), 2 min at 72 °C, with a final extension of 5–10 min at 72 °C. H3 amplification was performed with an initial denaturation for 3 min at 95 °C, followed by 40 cycles of 45–60 s at 94–95 °C, 45 s at 50 °C (annealing temperature), 2 min at 72 °C, with a final extension of 10 min at 72 °C.

Successful PCR products obtained at UCA were purified and sequenced by Macrogen, Inc. PCR products obtained at ZSM were purified using the NucleoSpin Extract II (Macherey-Nagel GmbH & Co). Cycle sequencing using Big Dye 3.1 and the PCR primers (10 pm/μl) was conducted by the Genomic Service Unit of the Department of Biology, Ludwig-Maximilians-University Munich. All new sequences obtained were deposited in GenBank.

Molecular analyses

DNA sequences were assembled and edited using Geneious v. 6.1.6 (Drummond *et al*., 2009). All the sequences were checked for contamination with BLAST (Altschul *et al*., 1990) implemented in the GenBank database. To align the sequences we used MAFFT (Katoh, Asimenos & Toh, 2009). The alignments were checked by eye using MacClade v. 4.06 (Maddison & Maddison, 2005). Protein-coding sequences were translated into amino acids for confirmation of alignment. Pairwise uncorrected p-distance values between each taxon were calculated for the COI gene using PAUP v. 4.ob10 (Swofford, 2002). Uncorrected p-distances between all taxa, and level of saturation for first, second and third codon positions (p-distances against transitions plus transversions) were calculated in MEGA v. 5.0 (Tamura *et al*., 2011) for the COI and H3 genes.

The most variable regions from the 16S rRNA alignment were removed in the first analyses, using both the default settings and the standard options for stringent and less stringent selection in Gblocks (Talavera & Castresana, 2007). When these regions were excluded from the analyses, the combined phylogenetic tree was poorly resolved with low nodal support. Therefore, final analyses were performed including all bases. Individual gene analyses and a concatenated analysis were performed. The best-fit models of evolution for each gene were determined using the Akaike information criterion (Akaike, 1974) implemented in MrModeltest v. 2.3 (Nylander, 2004). The GTR+I+G model was selected for the concatenated analysis.

Maximum likelihood (ML) analyses were performed using the software RAxML v. 7.0.4 (Stamatakis, 2006) and nodal support was assessed with nonparametric bootstrapping (BS) with 5000 replicates, random starting trees and parameters estimated from each dataset under the model selected for the original dataset. Bayesian inference analyses (BI) were conducted using MrBayes v. 3.1.2b (Ronquist & Huelsenbeck, 2003) for five million

Table 1. Specimens used in this study, with localities, museum voucher numbers and GenBank accession numbers (including the original museum voucher number, if available).

Species	Locality	Museum Voucher Number	COI	16S	H3
Tritonia challengeriana	Bouvet Island, Norway (EA)	CASIZ 171177 (GB)	HM162718.1	HM162643.1	HM162550.1
Piseinotecus gaditanus	Spain (EA)	MNCN 15.05/53704 (GB)	HQ616759	HQ616722	HQ616788
Spurilla neapolitana	Balearic Island, Spain (MED)	MNCN/ADN: 51961 (GB)	JX087582	JX087517	JX087655
Spurilla neapolitana	France (EA)	MNCN/ADN: 51969 (GB)	JX087574	JX087514	JX087650
Berghia verrucicornis	Morocco (EA)	MNCN 15.05/53686 (GB)	HQ616749	HQ616712	HQ616778
Berghia verrucicornis	Spain (EA)	MNCN 15.05/53687 (GB)	HQ616750	HQ616713	HQ616779
Triopha maculata	Marin County, Duxbury Reef, California, USA (EPAC)	CASIZ 181556 (GB)	HM162691.1	HM162601.1	HM162507.1
Triopha catalinae	San Francisco Yacht Harbor, San Francisco, USA (EPAC)	CASIZ 170648 (GB)	HM162690.1	HM162600.1	HM162506.1
Peltodoris nobilis	Pillar Point, San Mateo County, USA (EPAC)	CASIZ 182223 (GB)	EU982761	EU982816	HM162499.1
Noumea haliclona	Port Philip Bay, Australia (WPAC)	SAM D19269 (GB)	EF535117.1	EF534045.2	–
Felimare villafranca	Taghazout, Morocco (EA)	MNCN 15.05/70681	KJ911288	KJ911268	KJ911248
Felimare villafranca	Taghazout, Morocco (EA)	MNCN 15.05/70682	KJ911289	KJ911269	KJ911249
Felimare villafranca	Menorca, Cap Cavalleria, Spain (MED)	MNCN 15.05/70683	KJ911290	KJ911270	KJ911250
Felimare villafranca	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70684	KJ911291	KJ911271	KJ911251
Felimare villafranca	San García, Cadiz, Spain (Strait of Gibraltar)	MNCN 15.05/70695	KJ911292	KJ911272	KJ911252
Felimare villafranca	Ilhas do Martinhal, Algarve, Portugal (EA)	CASIZ 185127 (GB)	–	JQ727793.1	–
Felimare villafranca	Cadiz, Spain (EA)	GB	AJ223266.1	AJ225190.1	–
Felimare villafranca	Spain (EA)	GB	–	AF249237.1	–
Goniobranchus splendidus	Mooloolaba, Queensland, Australia (WPAC)	CASIZ 146039 (GB)	EU982738.1	EU982789.1	–
Goniobranchus splendidus	Mooloolaba, Queensland, Australia (WPAC)	SAM D19292 (GB)	EF535115.1	AY458815.1	–
Chromodoris strigata	Nosi Kalakjoro, Iles de Radama, Madagascar (IO)	CASIZ 175558 (GB)	JQ727857.1	JQ727739.1	–
Chromodoris strigata	Maricaban Island, Batangas, Philippines (WPAC)	CASIZ 158260 (GB)	JQ727856.1	JQ727738.1	–
Chromodoris aspersa	Mooloolaba, Queensland, Australia (WPAC)	SAM D19282 (GB)	–	AY458813.2	–
Chromodoris aspersa	Napili Bay, Maui, Hawaii (WPAC)	CASIZ 174975 (GB)	–	JQ727705.1	–
Chromodoris magnifica	Whitsundays, Queenslad, Australia (WPAC)	SAM D19290 (GB)	EF535110.1	EF534042.2	–
Chromodoris magnifica	Maricaban Island, Batangas, Philippines (WPAC)	CASIZ 157027 (GB)	EU982736.1	EU982787.1	–
Chromodoris magnifica	Mooloolaba, Queensland, Australia (WPAC)	CASIZ 144119 (GB)	JQ727852.1	JQ727731.1	–
Felimida edmundsi	Pedra Adalio, Príncipe Island, São Tomé and Príncipe (EA)	CASIZ 179385 (GB)	HM162686.1	HM162595.1	HM162501.1
Felimida edmundsi	Ilhéu Mosteiros, São Tomé and Príncipe (EA)	CASIZ 179394	KJ812351	KJ804240	KJ812364
Felimida edmundsi	Ilhéu Mosteiros, São Tomé and Príncipe (EA)	CASIZ 179411	KJ812352	KJ804241	KJ812365
Felimida edmundsi	Ilhéu Cabra, São Tomé (EA)	GB	EF535133.1	EF534061.2	
Felimida edmundsi	Azores, Portugal (EA)	ZMBN 81682	KJ812350	KJ804239	KJ812363
Felimida edmundsi	Azores, Portugal (EA)	ZMBN 81703	KJ812353	KJ804242	KJ812366
Felimida dalli	Santa Lucía Bay, Guerrero, Mexico (EPAC)	CNMO 4964	KJ911293	KJ911267	KJ911247
Felimida dalli	Guanacaste, Punta Carbon, Costa Rica (EPAC)	CASIZ 175428 (GB)	EU982741.1	EU982793.1	–
Felimida dalli	Tres Hermanas Island, Costa Rica (EPAC)	CASIZ 175439 (GB)	JQ727869.1	JQ727751.1	–
Felimida purpurea	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70693	KJ911285	–	KJ911244
Felimida purpurea	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70694	KJ911286	KJ911265	KJ911245
Felimida purpurea	Ilhéu dos Mosteiros, São Miguel Island, Azores, Portugal (EA)	ZMBN 87934	KJ812354	KJ804243	KJ812367
Felimida purpurea	Cadiz, Spain (EA)	GB	AJ223260.1	AJ225184.1	
Felimida krohni	Del Rey Island, Chafarinas, Spain (MED)	MNCN 15.05/70689	KJ911274	KJ911254	KJ911233
Felimida krohni	Congreso Island, Chafarinas, Spain (MED)	MNCN 15.05/70690	KJ911275	KJ911255	KJ911234
Felimida krohni	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70691	KJ911276	KJ911256	KJ911235
Felimida krohni	Guetaria Bay, Basque Country, Spain (EA)	MNCN 15.05/70697	KJ911277	KJ911257	KJ911237
Felimida krohni	Italy (MED)	MNCN 15.05/70698	KJ911278	KJ911258	KJ911236
Felimida krohni	Murcia, Spain (MED)	GB	AY345036.1	–	–
Felimida krohni	Spain (EA)	GB	AF249805.1	AF249239.1	–
Felimida luteorosea	Del Rey Island, Chafarinas, Spain (MED)	MNCN 15.05/70692	KJ911283	KJ911263	KJ911242
Felimida luteorosea	Guetaria Bay, Basque Country, Spain (EA)	MNCN 15.05/70696	KJ911284	KJ911264	KJ911243
Felimida luteorosea	Spain (MED)	GB	AF249815.1	–	–
Felimida luteorosea	Greece (MED)	NHMC 52.116	KJ812355	KJ804244	–
Felimida luteopunctata	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70685	KJ911279	KJ911259	KJ911238
Felimida luteopunctata	Santa Maria Beach, Cadiz, Spain (EA)	MNCN 15.05/70686	KJ911280	KJ911260	KJ911239
Felimida luteopunctata	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70687	KJ911281	KJ911261	KJ911240

Continued

Table 1. *Continued*

Species	Locality	Museum Voucher Number	COI	16S	H3
Felimida luteopunctata	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70688	KJ911282	KJ911262	KJ911241
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	MNCN 15.05/60113N	KJ812356	KJ804245	KJ812368
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	MNCN 15.05/60113	–	KJ804246	KJ812369
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	MNCN 15.05/69821	KJ812357	KJ804247	KJ812370
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	ZSM Mol 20130570	KJ812358	KJ804248	KJ812371
Felimida sphoni	La Unión Beach, Gulf of Fonseca, El Salvador (EPAC)	MHNES 90-0425	–	KJ804249	KJ812372
Felimida sphoni	Santa Lucía Bay, Guerrero, Mexico (EPAC)	CNMO 4965	KJ911287	KJ911266	KJ911246
Felimida sphoni	Herradura Beach, Punteras, Costa Rica (EPAC)	MZUCR8099	KJ812359	KJ804250	KJ812373
Felimida sphoni	Guanacaste, Punta Carbón, Costa Rica (EPAC)	CASIZ 175431 (GB)	–	JQ727736.1	–
Felimida baumanni	Guanacaste, Costa Rica (EPAC)	CASIZ 175434	KJ812360	KJ804251	KJ812374
Felimida baumanni	Reserva Natural Absoluta Cabo Blanco, Punteras, Costa Rica (EPAC)	MZUCR9023	KJ812361	KJ804252	–
Felimida baumanni	Tamarindo Beach, Guanacaste, Costa Rica (EPAC)	CASIZ 175433 (GB)	JQ727866.1	JQ727748.1	–
Felimida britoi	Ilhéu dos Mosteiros, São Miguel Island, Azores (EA)	ZMBN 87950	KJ812362	KJ804253	KJ812375
Felimida britoi	Madeira, Portugal (EA)	ZSM Mol 20130740	KJ911273	KJ911253	KJ911232

Abbreviations: EA, eastern Atlantic Ocean; EPAC, eastern Pacific Ocean; GB, GenBank; IO, Indian Ocean; MED, Mediterranean; WPAC, western Pacific Ocean. Asterisks indicate newly generated sequences.

generations with two independent runs and sampling frequency of 1000. The models implemented were those estimated with MrModeltest v. 2.3. The combined dataset was partitioned among genes and the 'unlink' command was used to allow all parameters to vary independently within each partition.

Convergence was diagnosed graphically by plotting for each run the likelihood against the number of generations using the software Tracer v. 1.4.1 (Drummond & Rambaut, 2007). For each analysis, the first 1250 trees were discarded as 'burn-in'. Nodal support was assessed with posterior probabilities (PP). Only nodes supported by BS ≥ 75 and PP ≥ 0.90 were considered as resolved.

Two species-delimitation analyses were made including *F. elegantula* and its closest related species *F. luteopunctata* (Gantès, 1962) and *F. luteorosea* (Rapp, 1827). The automatic barcode gap discovery (ABGD) method (Puillandre *et al.*, 2012) was performed using the online version of the software (available at http://wwwabi.snv.jussieu.fr/public/abgd/) with the default settings to generate a preliminary partition of sequences, using the COI alignment. In addition the species-delimitation plugin (Masters, Fan & Ross, 2011) in Geneious was used to provide a statistical framework to assess putative species in the phylogenetic analyses, using the Bayesian concatenated tree without modifications, with special interest in the same species.

RESULTS

Molecular results

The combined dataset based on COI, H3 and 16S yielded a sequence alignment of 1477 positions. No saturation was observed across genes and codon positions, not even in the third codon position (not shown). The resulting combined tree provided better resolution than H3, COI or 16S separately (not shown). Figure 1 shows the phylogenetic hypothesis based on the combined dataset constructed by Bayesian Inference. The topology of the ML tree was identical (not shown). Chromodorididae are monophyletic with high support in both Bayesian and ML analyses (PP = 1, BS = 86), but the relationships between species and genera were not well resolved (Fig. 1). Within Chromodorididae there is a polytomy consisting of: *Noumea haliclona* (Burn, 1957); *Felimare villafranca* (Risso, 1818); *Goniobranchus splendidus* (Angas, 1864); a clade of *Chromodoris magnifica* (Quoy & Gaimard, 1832), *C. strigata* Rudman, 1982 and *C. aspersa* (Gould, 1852) (PP = 1, BS = 100); *Felimida baumanni* (Bertsch, 1970); a clade including

Felimida edmundsi (Cervera, García-Gómez & Ortea, 1989) and *Felimida dalli* (Bergh, 1879) (PP = 1, BS = 85; a clade including *Felimida krohni* (Vérany, 1846) and *F. purpurea* (Risso in Guérin, 1831) (PP = 1, BS = 94); and a clade containing the remaining species of *Felimida* included in this study. Philippi's *Doris elegantula* nested in this last clade together with the type species of the genus, *Felimida sphoni* Marcus, 1971. *Felimida elegantula*, *F. luteopunctata* and *F. luteorosea* were retrieved in a common clade (PP = 1, BS = 100). Apart from differences in morphology and external colour pattern, the analysis using the species-delimitation plugin (Masters *et al.*, 2011) in Geneious confirmed *F. elegantula*, *F. luteopunctata* and *F. luteorosea* as distinct species. For this clade, 'P ID (Liberal)' minimum values were ≤ 0.91, i.e. a more than 90% chance of correctly placing an unknown specimen in its *a priori* designated species. For *F. elegantula* and *F. luteopunctata* these values were higher, ≤ 0.97 and ≤ 0.98, respectively (Table 2). The ABGD analysis recovered nine partitions with three groups each: one for *F. elegantula*, one for *F. luteopunctata* and one for *F. luteorosea* specimens. The prior maximal distance (P) ranged between 0.001 and 0.03. All tree topologies (from concatenated and single-gene analyses) supported monophyly of *F. elegantula*. The minimum uncorrected p-distances for COI between the *Felimida* species was 5.01% (*F. elegantula* – *F. luteorosea*) and the maximum 21.58% (*F. sphoni* – *F. purpurea*) (Table 3). Distances between the outgroup species *T. challengeriana* and the species of Chromodorididae ranged from 19.69% (*F. edmundsi*) to 23.58% (*F. purpurea*) (data not shown).

SYSTEMATIC DESCRIPTION

Chromodorididae Bergh, 1891

***Felimida* Ev. Marcus 1971**

Diagnosis: Unicuspidate lateral teeth, many denticles on outer side, denticles on the inner side of the lateral teeth 1–4 (Marcus, 1971).

***Felimida elegantula* (Philippi, 1844)**
(Figs 2–4)

Doris elegantula Philippi, 1844: 80, pl. 19, fig. 8.
Glossodoris elegantula—Pruvot-Fol, 1932: 327.
Chromodoris elegantula—Cattaneo-Vietti & Barletta, 1984: 205.
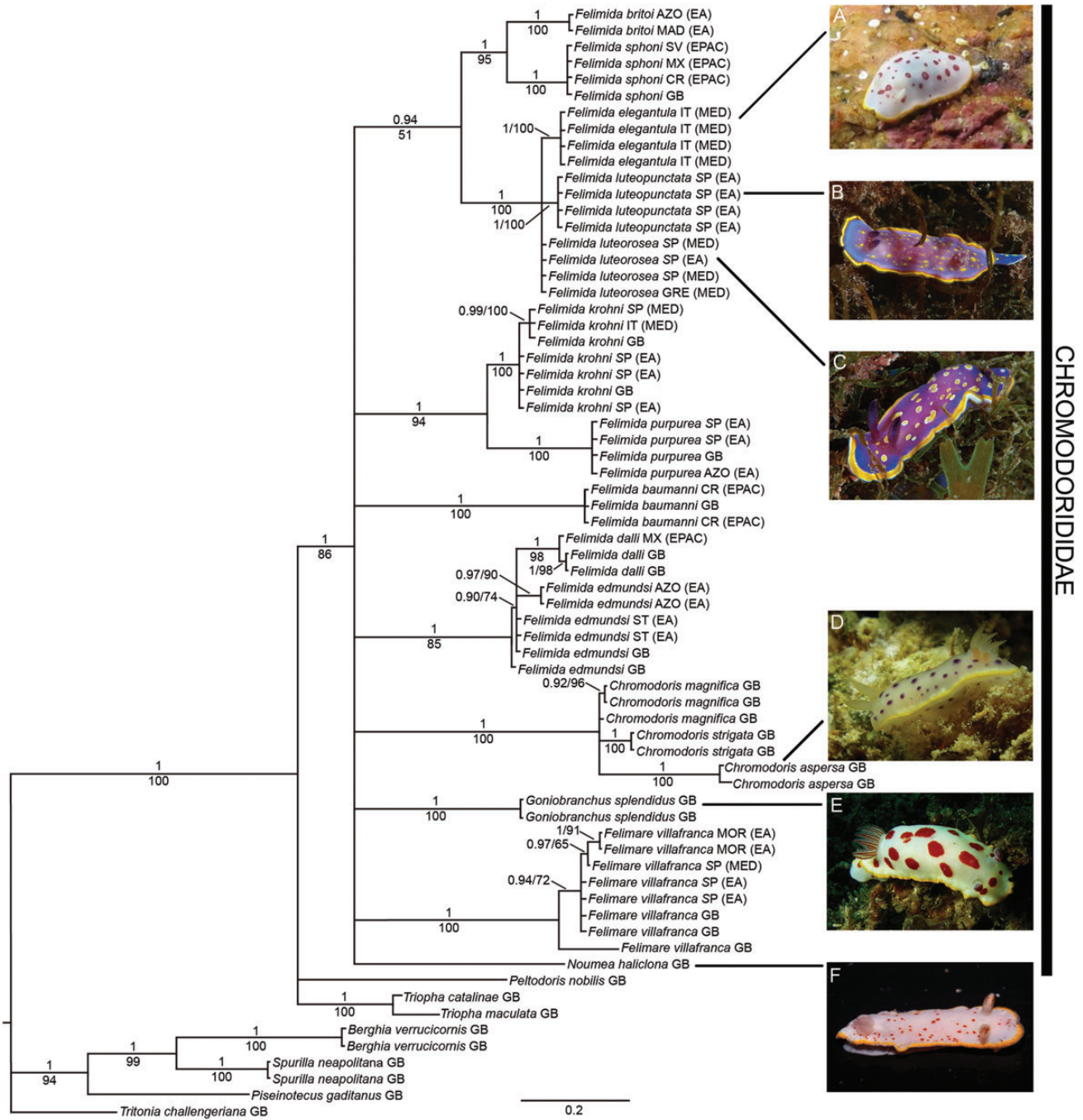
Figure 1. Phylogenetic hypothesis based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers above branches are posterior probabilities. Numbers below branches are bootstrap values. Abbreviations: ATL, Atlantic Ocean; AZO, Azores; CR; Costa Rica; GB, GenBank, GRE, Greece; IT, Italy; MAD, Madeira; MED, Mediterranean; MX, Mexico; MOR, Morocco; EA, Eastern Atlantic; EPAC, Eastern Pacific; SP, Spain; ST, São Tomé; SV, El Salvador. Photographs: **A.** *Felimida elegantula* (MNCN 15.05/60113; photo by E. Trainito). **B.** *F. luteopunctata* (photo by M. Martínez Chacón). **C.** *F. luteorosea* (photo by M. Martínez Chacón). **D.** *Chromodoris aspersa* (photo by S. Kahlbrock). **E.** *Goniobranchus splendidus* (photo by S. Kahlbrock). **F.** *Noumea haliclona* (photo by D. Aston).

Table 2. Species delimitations results for *Felimida* species, based on Bayesian analysis of concatenated sequences.

Species	Closest species	Monophyly	Intra Dist	Inter dist-closest	Intra/inter	P ID (strict)	P ID (liberal)
F. elegantula	*F. luteorosea*	Yes	0.003	0.060	0.06	0.83	0.97
F. luteopunctata	*F. luteorosea*	Yes	0.001	0.051	0.03	0.85	0.98
F. luteorosea	*F. luteopunctata*	Yes	0.014	0.051	0.28	0.68	0.91

Table 3. Minimum and maximum pairwise uncorrected p-distances for COI between chromodorid species.

Species	Felimida britoi (EA)	Felimida sphoni (EPAC)	Felimida elegantula (MED)	Felimida luteopunctata (EA)	Felimida luteorosea (EA, MED)	Felimida krohni (MED)	Felimida purpurea (EA)	Felimida baumanni (EPAC)	Felimida dalli (EPAC)	Felimida edmundsi (EA)	Chromodoris magnifica (WPAC)	Chromodoris strigata (WPAC)	Goniobranchus splendidus (WPAC)	Felimare villafranca (EA, MED)	Noumea haliclona (WPAC)
F. britoi	0														
F. sphoni	15.2–15.6	0													
F. elegantula	16.1–16.3	18.1–18.4	0												
F. luteopunctata	16.4–16.9	18.4–18.5	6.7–7.0	0											
F. luteorosea	15.1–16.9	17.5–18.4	5.0–6.0	5.3–5.8	0										
F. krohni	17.5–19.0	17.9–18.6	15.7–17.3	17.2–17.8	15.7–18.1	0									
F. purpurea	19.0–20.1	20.6–21.6	18.4–20.1	16.9–18.4	16.4–18.7	15.5–17.8	0								
F. baumanni	16.7–17.0	18.2–19.6	17.6–18.1	17.9–18.4	16.9–17.9	16.9–17.8	18.8–20.4	0							
F. dalli	18.4–19.1	18.8–20.2	16.3–17.3	17.6–17.8	16.1–17.3	15.2–17.3	17.5–19.4	16.0–16.6	0						
F. edmundsi	17.2–17.8	17.6–18.9	15.2–16.3	17.0–17.8	15.2–16.7	15.0–16.9	18.9–20.2	16.3–17.2	7.4–8.3	0					
C. magnifica	16.8–18.1	17.8–20.5	16.9–17.6	16.7–17.0	15.8–17.5	15.8–17.3	17.8–18.8	15.9–17.6	16.7–17.8	17.2–18.2	0				
C. strigata	17.6–18.1	19.0–20.2	17.8–18.1	17.0–17.3	16.9–17.5	17.9–18.8	19.3–19.9	18.1–18.4	17.3–17.5	17.9–18.8	6.8–7.7	0			
G. splendidus	17.1–18.1	16.4–18.4	14.6–15.6	15.2–16.0	15.8–16.7	17.0–18.1	16.0–17.0	17.8–18.2	14.9–15.8	14.8–15.4	16.4–17.1	16.1	0		
F. villafranca	17.6–19.7	18.7–19.6	17.5–17.9	17.0–18.2	16.9–18.1	17.4–19.4	18.0–19.6	19.6–20.8	15.4–17.8	15.2–16.9	16.9–19.4	16.8–18.7	17.6–19.6	0	
N. haliclona	15.9–16.1	16.8–17.4	16.3	17.3	16.6–17.5	16.0–17.0	17.2–18.4	18.2–18.4	16.1–16.7	15.4–15.8	19.7–21.0	20.7–20.8	16.3	16.3–17.5	0

Abbreviations: MED, Mediterranean; EA, Eastern Atlantic; WA, Western Atlantic; EPAC, Eastern Pacific; WPAC, Western Pacific.

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Figure 2. Living specimen of *Felimida elegantula*. Porto San Paolo, northeastern Sardinia, Italy. (MNCN 15.05/60113; photo by E. Trainito).

Type material: Not located and believed lost. Thus, we designate here as neotype the specimen MNCN 15.05/60113N (preserved length 14 mm, dissected; 4–5 m depth, 10 Dec. 2011, Porto San Paolo, Sardinia, Italy, coll. E. Trainito).

Material examined: 5 specimens, Porto San Paolo, northeastern Sardinia, Italy: MNCN 15.05/60113N (10 Dec. 2011; preserved length 14 mm, dissected); MNCN 15.05/60113 (10 Dec. 2011; 9 mm); MNCN 15.05/60113 (10 Dec. 2011; 10 mm, dissected); ZSM Mol 20130570 (10 Oct. 2013; 5 mm); MNCN 15.05/69821 (10 Oct. 2013; 7 mm, dissected).

External morphology (Fig. 2): Body oval, elongate. Living animals white with several small red spots irregularly covering notum, lateral side of foot and tail; opaque white patches over notum; mantle edge yellow with thin opaque white band on inner side. Posterior end of foot not covered by notum. Mantle dermal formations (MDFs) not very conspicuous around mantle edge, absent at anterior region. Rhinophores with up to 13 lamellae. Gill with six pale white unipinnate branchial leaves, each with opaque white rachis. Completely retractile rhinophores and gills.

Internal anatomy (Fig. 3): Radular formulae of three specimens: $38 \times 31.1.31$ (MNCN 15.05/60113N, 14 mm), $34 \times 20.1.20$ (MNCN 15.05/69821, 7 mm); $29 \times 27.1.27$ (MNCN 15.05/60113, 10 mm). Rachidian teeth small but clearly visible, triangular, without denticulation (Fig. 3A). Innermost lateral teeth bifid; inner cusp broad; outer cusp slightly curved downwards with 5–7 denticles (Fig. 3A). Median lateral teeth with 5–8 denticles on inner face (Fig. 3B). Outermost lateral teeth with reduced broad base and 7–10 denticles at tip of each tooth (Fig. 3C). Labial cuticle brown, generally with bifid rodlets, but sometimes simple and bifid rodlets equally distributed (Fig. 3D).

Reproductive system (Fig. 4D) hermaphroditic, triaulic. Anterior portion occupying a relatively small space between buccal mass and digestive gland. Hermaphrodite duct flattened and short; ampulla moderately long, thin, centrally folded
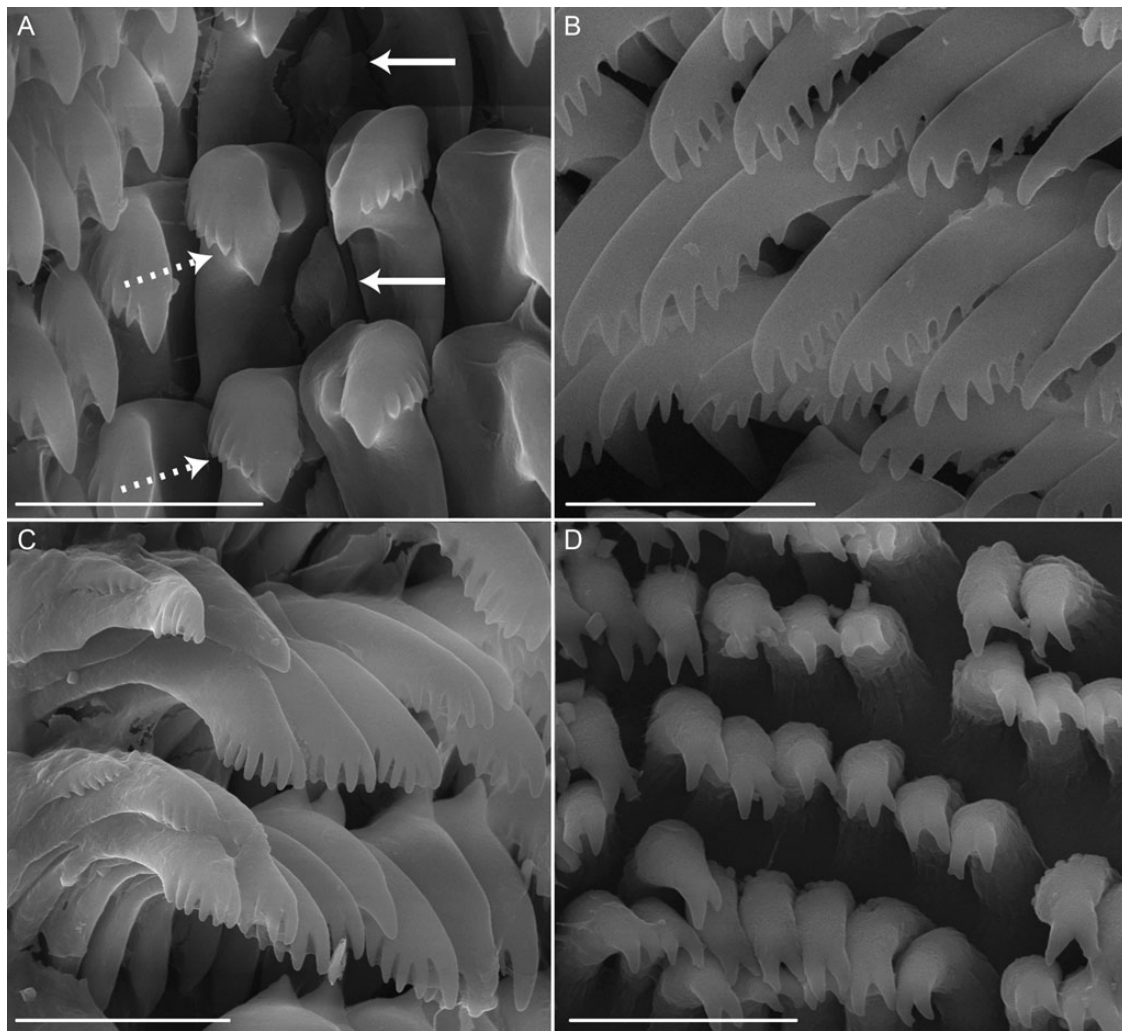
Figure 3. Scanning electron microscopes of *Felimida elegantula*. **A.** Detail of rachidian teeth (arrows) and denticles of the innermost lateral teeth (dashed arrows) (MNCN 15.05/69821). **B.** Median lateral teeth (MNCN 15.05/69821). **C.** Outermost lateral teeth (MNCN 15.05/69821). **D.** Elements of the armature of the labial cuticle (MNCN 15.05/69821). Scale bars: **A, B, C** = 25 μm; **D** = 12.5 μm.

(Fig. 4B, C). Prostate elongated, located ventrally to bursa copulatrix; deferent duct long, with many folds; transition between deferent duct and penial portion well demarcated (Fig. 4C); penial portion wide, located ventral to female gland and vagina, lacking any of accessories such as penial spines or glands. Vagina wide, moderately long (Fig. 4A, B); seminal receptacle small, pyriform (Fig. 4B). Bursa copulatrix very large, rounded (Fig. 4A). Uterine duct moderately long, large (Fig. 4B), inserting into female gland mass near to oviduct. Oviduct short (Fig. 4C). Female gland mass small, nidamental region with a rounded portion, ventral to vagina. Vestibular gland near orifice of female gland (Fig. 4A–C).

Geographical distribution (Fig. 5): *Felimida elegantula* has rarely been recorded since its original description (Philippi, 1844). It is only known from the Mediterranean Sea and has been recorded in Spain: Malgrats Islands (Balearic Islands) (Vives, 2007); France: Villafranche-sur-Mer (Pruvot-Fol, 1932); Italy: Palermo (Sicily) (Philippi, 1844, type locality), Porto San Paolo (present study), Cagliari and Tavolara (Sardinia) (Trainito, 2003; Piras, 2005), Secche della Meloria (Sordi, 1970), Pantelleria Island (Picchetti, 2000); Maltese archipelago (Cachia, Mifsud & Sammut, 1993; Perrone, 1993; Sammut & Perrone, 1998); and Turkey: Adrasan (Türkmen & Demirsoy, 2009).

DISCUSSION

Felimida elegantula was described by Philippi (1844) based on a single specimen from Sicily. The holotype was described as having a rectangular, opaque white body with dorsal small dark spots, yellow mantle edge, 11 branchial leaves and white rhinophores (Philippi, 1844). The colour pattern of our specimens from Porto San Paolo resembles Philippi's description, but also Perrone's (1993) *F. elegantula polychroma* regarding the size of the spots. According to this last author, the external differences between his two subspecies, *F. elegantula elegantula* and *F. elegantula polychroma*, were the larger spots and the presence of blotches in the latter. Since no specimens matching Perrone's description of *F. elegantula elegantula* are available for molecular analysis, it is not possible to test if his subspecies are intraspecific variants or different species.

Regarding the internal anatomy, Philippi (1844) did not present any data in the original description. Subsequently, Pruvot-Fol (1932, 1954), Sordi (1970) and Perrone (1993) reported some information about the number of radular teeth per row, but only Perrone (1993) gave a complete radular formula for *F. elegantula polychroma*, reporting 48 rows in one 27 mm specimen (48 × 52.0.52). None of these authors clearly stated the presence of a rachidian tooth, although Sordi (1970:
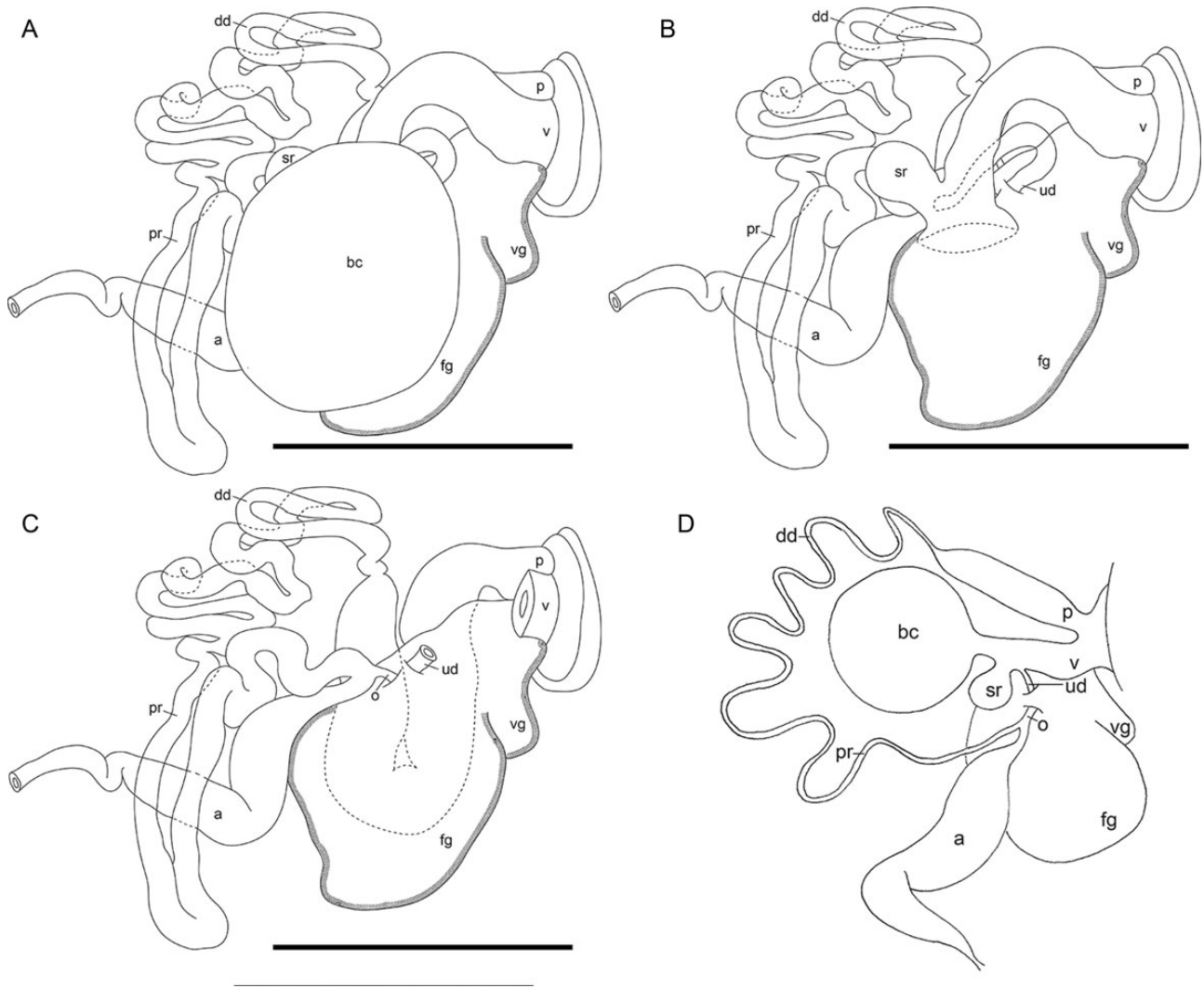
Figure 4. Reproductive system of *Felimida elegantula* (MNCN 15.05/60113N). **A.** Complete reproductive system. **B.** Partial view of reproductive system; bursa copulatrix removed. **C.** Partial view of reproductive system; bursa copulatrix, vagina, receptaculum seminis and uterine duct removed. **D.** Schematic drawing of complete reproductive system. Scale bars = 1.0 mm. Abbreviations: a, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland mass; o, oviduct; p, penis; pr, prostate; sr, receptaculum seminis; ud, uterine duct; v, vagina; vg, vestibular gland.

24) mentioned a "dente mediano quasi impercettibile", while giving the formula 50–0–50, and Perrone (1993: 75) stated "rachidiano è ridotto ad un semplice ispessimento, scarsamente visibile". Cattaneo-Vietti *et al*. (1990) first gave a radular formula including a rachidian tooth (38 × 35.1.35), which was described as subtriangular and evident. This description matches the rachidian teeth of our specimens. Concerning the armature of the labial cuticle, Perrone (1993) depicted the elements as curved and bifid, but in our specimens there are two different elements, some bifid and some with only one cusp. Perrone (1993) also stated that *F. elegantula polychroma* has the same kind of elements as *F. elegantula elegantula*.

The only information given by Sordi (1970) about the reproductive system was that the penis was unarmed. Perrone (1993) also mentioned that the penes of *F. elegantula elegantula* and *F. elegantula polychroma* were unarmed and that the female gland of the latter was easily separated from the bursa copulatrix and the deferent duct. This was also observed in the specimen studied in the present work (MNCN 15.05/60113N). Ours is the first study to give a complete and detailed description of the reproductive system of *F. elegantula*. The presence of a vestibular gland was not described by Perrone (1993), but it was observed in other species

of the genus, e.g. in *F. luteopunctata* (Cervera, García-Gómez & Ortea, 1989), *F. luteorosea* (García-Gómez, 2002), and *Felimida corimbae* (Ortea, Gofás & Valdés, 1997).

Felimida elegantula clearly differs from other chromodorids in the Mediterranean Atlantic, by its unique and characteristic colour pattern and morphological features. The rachidian tooth is present in other Mediterranean species such as *F. britoi* (Ortea & Pérez, 1983), *F. luteopunctata* and *F. luteorosea*, while in *F. purpurea* it was observed by Cattaneo-Vietti *et al*. (1990), but not by García-Gómez (2002). The reproductive system of the species in the clade of *F. luteopunctata*, *F. luteorosea* and *F. elegantula* (Fig. 1) is quite similar, but with differences in the width and length of the vagina and in the shape of the receptaculum seminis. In *F. elegantula* the vagina is very wide and short, while in *F. luteopunctata* and *F. luteorosea* it is short but thin (Cervera *et al*., 1989; García-Gómez, 2002). The vagina of *F. britoi* is also short and thin (Ortea & Pérez, 1983). *Felimida purpurea* and *F. krohni* have a thin and very elongated vagina (García-Gómez, 2002). In addition, the receptaculum seminis in *F. elegantula* is quite different from other Atlantic *Felimida* as it is almost rounded, while in others such as *F. luteopunctata*, *F. luteorosea*, *F. purpurea*, *F. britoi* and *F. krohni* it is elongated and sausage-shaped (Ortea & Pérez,

Figure 5. Distribution of *Felimida elegantula* according to published literature and photographs (see text).

1983; Cervera *et al.*, 1989; García-Gómez, 2002). Two externally similar *Felimida* species from Angola, *F. corimbae* and *F. ocellata* (Ortea, Gofás & Valdés, 1997), also show differences from *F. elegantula* since both have a short but thin vagina and an elongated receptaculum seminis (very long in *F. corimbae*) (Ortea *et al.*, 1997). The validity of *F. elegantula* as a distinct and valid species is supported by the monophyly of our newly collected specimens shown in the single and combined gene trees and by the species delimitation analyses.

In their molecular study, Johnson & Gosliner (2012) included nine putative *Felimida* species from the known distribution for this genus and, although these did not form a monophyletic clade based on mitochondrial COI and 16S markers, they nevertheless re-erected this genus. Our extended dataset includes 10 *Felimida* species with 32 additional specimens and additional sequences which include a nuclear gene for the first time (34 new sequences for COI, 35 for 16S gene, 34 for H3). Nevertheless, analyses of our combined dataset still did not recover *Felimida* as monophyletic, instead showing a polytomy with other chromodoridid groups, corroborating the previous ambiguous results. For now, we have decided to allocate *Doris elegantula* to *Felimida*, since the species clusters in the same clade as the type species, *F. sphoni*, from the eastern Pacific. A better resolution of the phylogeny of the eastern Pacific and Atlantic chromodoridids awaits comprehensive sampling of species from throughout these regions.

It is notable that in our study *F. elegantula* clustered together with the two other spotted *Felimida* species from the Atlantic (*F. luteopunctata* and *F. luteorosea*) (Fig. 1), suggesting that, regardless of the colour, the spotted pattern of these chromodoridids may have a common origin. The close relationship between *F. krohni*, *F. purpurea* and *F. luteorosea* shown by Johnson & Gosliner (2012) was not recovered in our study nor in that by Valdés *et al.* (2011). Interestingly, Rudman (1983) remarked on the external resemblance of *F. elegantula* to some Indo-Pacific species such as *Goniobranchus splendidus* (as *Chromodoris splendida*) and *Chromodoris aspersa* (Fig. 1), grouping them in his 'Chromodoris splendida colour group'. Ortea *et al.* (1997) also noted the similarity in colour of *F. elegantula*, *F. ocellata* (as *Glossodoris ocellata*) and *F. corimbae* (as *Chromodoris corimbae*) with Indo-Pacific species of Rudman's *Chromodoris splendida* colour group. However, Rudman (1983) also commented that it would be unlikely that species with such wide

geographic separation (Mediterranean and Indo-west Pacific) could be closely related. Our results corroborate Rudman's statement, with Indo-Pacific species clustering together and distant from Atlantic and Mediterranean ones.

The uncorrected p-distances for COI between members of *Felimida* ranged from 5.01% to 21.58%, some of them high if compared with typical interspecific values (mean $11.2 \pm 5\%$) (Hebert, Ratnasingham & Waard, 2003), and with those found between other heterobranch species [7% between two '*Glossodoris*' species (Valdes *et al.*, 2011); 10% for *Bulla* (Malaquias & Reid, 2008); 10–20% for sacoglossans (Krug, Händeler & Vendetti, 2011)].

In order to resolve these phylogenetic questions the dataset for *Felimida* (*sensu* Johnson & Gosliner, 2012) should be increased to include as many species as possible, in particular *F. macfarlandi* (Cockerell, 1902) from the eastern Pacific, *F. rolani* (Ortea, 1988) from the eastern Atlantic and *F. grahami* (Thompson, 1980) and *F. binza* (Ev. Marcus & Er. Marcus, 1963) from the tropical western Atlantic.

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3. RESULTS

<u>Chapter 11</u>

Padula V, Bahia J, Stöger I, Camacho-García Y, Malaquias M, Cervera JL, Schrödl M. (in prep.). A test for color based taxonomy in nudibranchs: molecular phylogeny and species delimitation of the *Felimida clenchi* (Mollusca: Chromodorididae) species complex. *Molecular Phylogenetics and Evolution*.

A test for color based taxonomy in nudibranchs: molecular phylogeny and species delimitation of the *Felimida clenchi* (Mollusca: Chromodorididae) species complex

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ABSTRACT

1. Introduction

Nudibranchs are one of the most colorful and diverse groups of marine invertebrates. Similarly to what occurs to most chromatic animal groups, as butterflies, frogs and reef fish, color is used as a diagnostic character to separate species, or groups of species, of nudibranchs. In fact, external morphology and color patterns are the most generally features for the identification of the species, as can be seen in many specialized guide books (Valdés *et al.* 2006, Debelius *et al.*, 2008, Gosliner *et al.* 2008). The Chromodorididae is the largest family among dorid nudibranchs, with more than 300 described species and geographically distributed primarily in tropical and subtropical seas (Turner & Wilson, 2007, Johnson & Gosliner, 2012). As the name suggests, most chromodorids are brightly colored, being a favorite topic for underwater macro photographers. They are among the most colorful nudibranchs, ranging from millimeters to few centimeters in length and living mostly between rocks in intertidal and subtidal zones. Chromodoridids feed on sponges and each genus seems to be specialist predator on one or few sponge families (Rudman & Bergquist, 2007). From the sponges nudibranchs can obtain, accumulate and also transform toxic compounds to be used in their own defense. Due to this property, many species were also chemically studied (Cimino & Ghiselin, 2009). Diversity of forms and species richness are high in the Indo-Pacific region, where many species groups with similar color pattern and morphology were proposed and described (Rudman 1982, 1983, 1985,

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1986a,b, 1987, 1990, 1991). The number of species is considerable smaller in the Atlantic Ocean and the Mediterranean Sea. However, some highly charismatic species color groups are also present in these regions, such as the group of blue *Felimare* species distributed mostly in the Eastern Atlantic and the Mediterranean Sea (Ros, 1976, Ortea *et al.*, 1996). In the last years, first molecular studies focused on chromodoridid species were published. Turner & Wilson (2007) recovered evidence of paraphyly or polyphyly in the different widespread genera examined, a scenario which was later confirmed and further examined with the addition of more species (Johnson & Gosliner, 2012). The last authors recovered available old names for new clades identified in their phylogenetic hypothesis. *Chromodoris*, the type genus of the family, for example, is not a widespread genus in the tropical and subtropical zones of all oceans. Species from the Eastern Pacific, Atlantic and Mediterranean Sea are part of the genus '*Felimida*'. The monophyly of *Felimida* still needs to be tested with a more comprehensive analysis (Johnson & Gosliner, 2012; Ortigosa *et al.* 2014) but this generic name is now commonly used by the scientific community.

1.1 The Felimida clenchi *species complex*

The *Felimida clenchi* species complex represents a history of exceptional controversy and discussion among expert taxonomists (Meyer, 1977, Thompson, 1980, Edmund & Just, 1985, Ortea & Pérez, 1983, Gosliner, 1990, Ortea *et al.* 1994). Original scanty, black and white descriptions potentially contributed to this scenario. Four nominal species are included in the complex: *Felimida clenchi* (Russell, 1935), *Felimida neona* (Marcus, 1955), *Felimida binza* (Marcus & Marcus, 1963) and *Felimida britoi* (Ortea & Pérez, 1983). *Felimida clenchi* was described based on a single nine millimeters long specimen found in Bermuda. The description includes simple drawings of the specimen in ventral and dorsal position; from the latter it is possible to see the dorsal opaque-white spots and the white submarginal band described as characteristic of the species (Russell, 1935). No internal structures were described. *Felimida neona* was described based on two specimens from São Paulo, southeastern Brazilian coast, as easily distinguishable from *F. clenchi* by the absence of dorsal oval or circular opaque-white spots and the presence of fluorescent red lines on the mantle (Marcus, 1955). The original description of *Felimida binza*, based on two specimens from Curacao, indicates a species similar to *F. clenchi*, but with occurrence of thin transversal dark dashes at the margin of the mantle, as can be seen in the original figure (Marcus & Marcus, 1963, fig. 30). Marcus & Marcus (1963) did not cite or make taxonomic comparison between *F. binza* and *F. clenchi*. Marcus & Marcus (1967a) studied three different specimens from Florida. Two of them (Marcus & Marcus, 1967a, Figs 58 and 58A) clearly agree to the original descriptions and illustrations of *F. clenchi* and *F. binza*, respectively, but unexpectedly the authors grouped all under the name *F. neona*, without further discussion. Meyer (1977), based on many specimens collected in the coast of Panama, synonymized *F. neona* to *F. clenchi*. He commented that *F. clenchi* is highly variable in color and internal morphology;

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this decision was followed by Thompson (1980) and Edmund & Just (1985) in their studies based on specimens from Jamaica and Barbados, respectively.

An additional species was described later from the Canary Islands by Ortea & Pérez (1983): *Felimida britoi* (Ortea & Pérez, 1983), a species with purple mantle with dorsal yellow or white lines and spots. According to Ortea & Perez (1983), *F. britoi* is similar to *F. neona* but presents a different color pattern, specimens are larger and with differences in the radular morphology when compared to the western Atlantic species. Studying specimens encountered in Azores, Gosliner (1990) considered *F. neona* and *F. britoi* synonyms of *F. clenchi*, considering the latter as "one of the most variable species of chromodorids in terms of its coloration". Due to the problematic history of this group of species, Ortea *et al.* (1994) conducted a morphological revision including the different color morphotypes. However, the authors attributed to *F. binza* and *F. clenchi* color morphotypes that are the inverse of what is presented in the original description and illustration of these species. For example, they named *F. clenchi* as species with many dorsal circular spots and submarginal dashes on the mantle, a characteristic not present in the original description of *F. clenchi* (Russell, 1935, plate 4), but clearly observable in the description of *F. binza* (Marcus & Marcus, 1963, fig. 30). The revision of Ortea *et al.* (1994) considered valid the four nominal species: *F. clenchi, F. neona*, *F. binza* and F. *britoi*, each with a diagnostic color pattern (Figure 1) and with small differences in the radula, reproductive system morphology and geographic distribution. Current classification and species identification are based mostly on this revision of Ortea *et al.* (1994), as can be seen published in articles and specialized websites published since them (Rudman 2000a,b, Domínguez *et al.*, 2006, Padula *et al.*, 2012, Sales *et al.* 2013, Camacho-García *et al.* 2014).

1.2 Color on nudibranchs

For a human eye, there are two main sources of color when seeing a nudibranch: the color of its internal structures and substances visible through a translucent mantle; or a true pigmentation of the mantle cells. It is already known that some species are variable in color accordingly to the color of their food and their life stage, as is the case of some aeolid nudibranchs. A *Favorinus* species, which feeds on the egg masses of other sea slugs, can be polychromatic if, in different events, it feeds on eggs with different colors. For *Spurilla*, body color generally depends on the color of the sea anemone on it feeds regularly. Recent molecular study confirmed the variability in the color pattern within species of the genus (Carmona *et al.* 2014). On the other hand, small fixed differences on a same general color pattern can indicate the occurrence of cryptic species, as is the case of *Cratena* (Padula *et al.* 2014). Regional color pattern differences were recorded within dorid nudibranchs with faded colors, like *Doriopsilla* (Goodheart & Valdés, 2012). For bright colored species, such as the Chromodorididae, it is clear that the different colors of the mantle are not directly derivate from the color of their food or their life stage, but the mechanisms acting for these different color patterns are

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still unknown (Ros, 1976, Edmunds, 1987). It is generally believed that the color pattern is not extreme variable within bright colored chromodoridid species. Rudman (1991), in his study on the purpose of pattern and the evolution of color in Chromodorididae, based on many representatives from Australia, stated that the patterns are very stable within a species and "when, in rare cases, species have a variable colour pattern, the variation is usually expressed as a number of quite distinct colour morphs, which are usually allopatric". The hypotheses of Rudman (1981) on Indo-Pacific species were not tested through molecular studies, but recent studies on Caribbean *Felimare* species (Ortigosa & Valdés, 2012) and Atlantic *Felimida* (Ortigosa *et al.* 2014, Padula *et al.* 2014) corroborate the idea of stable body color patterns within chromodorid species.

1.3 Objectives

The absence of comprehensive studies, using molecular data, dedicated to species delimitation of Chromodorididae and other chromatic nudibranch groups leaves untested the hypotheses of body color pattern as always reliable character for species identification. The confused taxonomic history of the *F. clenchi* group points to the possibility that, in some cases, body color may represent a more variable character within nudibranch species. In recent surveys in the Caribbean Sea and Brazil we observed morphotypes of *F. clenchi* and *F. binza* (*sensu* Ortea *et al.*, 1994), and *F. binza* and *F. neona* (*sensu* Ortea *et al.*, 1994) occurring in syntopy. On the other hand, observed small but fixed differences among specimens with the same general pattern, as the 'dorsally circular spotted' form attributed to *F. clenchi,* could indicate the existence of different species. From the data available in the literature and our field observations, we herein test two, almost contradictive, hypotheses on the *F. clenchi* group of species 1) The "different can be the same" hypothesis: existence of species with variable color pattern that would overlap with other species limits according to current color-based taxonomy, and; 2) The "equal can be different" hypothesis: specimens with a same general color pattern, but presenting small fixed differences, being part of different species. Based on specimens from different regions of the four nominal (*F. clenchi*, *F. neona*, *F. binza*, *F. britoi*) species geographic distribution we obtained comparative evidences from mitochondrial (COI and 16S) and nuclear (H3 and partial 28S) markers in phylogenetic, species delimitation (ABGD, Puillandre *et al.*, 2012; GMYC, Monaghan *et al.* 2009, Pons *et al.* 2006; PTP, Zhang *et al.* 2013) and haplotype network analyses (TCS, Clement *et al.*, 2000), in addition to the study of the reproductive morphology. Different evidences can guide toward different conclusions about the total number of species on delimitation studies (Padial *et al.* 2010, Jörger *et al.* 2012, 2014, Krug *et al.* 2013). Analyzing the number and weight of the different evidences obtained, we herein present a new hypothesis on the *F. clenchi* species group diversity.

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2. Material and methods

2.1 Taxon sampling

We aimed to include specimens covering the range of color morphotypes which could be part of the *Felimida clenchi* complex. Type specimens of the *F. clenchi*, *F. neona*, *F. binza* and *F. britoi* were searched in scientific collections, the holotypes of *F. binza* and *F. britoi* were located but could not be sequenced. The new samples cover most of the known geographic range of the four species, with the inclusion also of new localities, such as Saint Helena Island, in the South Atlantic Ocean. Specimens came from a joint collecting effort by the authors and collaborators during recent years. Specimens (Table 1) were collected manually and directly through free and scuba diving, down to 30 meters depth. Additionally, specimens of *Felimare kempfi* (Marcus, 1971), *Felimida krohni* (Vérany, 1846) and *Felimida sphoni* Marcus, 1971 were also collected for inclusion in the phylogenetic analyses. *Felimare kempfi* was selected as outgroup. The inclusion of *F. sphoni* is justified as this species resulted sister to *F. clenchi* and *F. binza* in previous molecular phylogenetic analysis (Johnson & Gosliner, 2012; Ortigosa *et al.*, 2014). The color pattern of *F. krohni* resembles that of *F. britoi*, and both species occur in sympatry, so it was included to evaluate relationships to the members of the *Felimida clenchi* complex. All specimens were photographed individually alive and were preserved in 70% or 96% EtOH. Studied material is deposited at Museu de Zoologia da Universidade de São Paulo, Brazil (MZSP); the Zoologische Staatssammlung München, Germany (ZSM); Museo de Zoología Universidad de Costa Rica (MZUCR); Natural History Museum of Crete, Greece (NHMC); Colección Nacional de Moluscos, Universidad Nacional Autónoma de México (CNMO); Museu Municipal do Funchal (História Natural), Portugal (MMF HN); and in the invertebrate collection of the California State Polytechnic University, Pomona (CPIC). Sequences of one specimen of *Felimida norrisi* (Farmer, 1963) and one specimen of *Felimida clenchi* were obtained from the GenBank. A list of all specimens used in the present study is disposed in Table 1.

2.2 DNA extraction, amplification and sequencing

Genomic DNA of each specimen was extracted from a small foot fragment using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co), following the manufacturer's instructions. Four markers were amplified through polymerase chain reaction (PCR) using universal primers: Cytochrome c Oxidase subunit I (COI), using primers from Folmer *et al.* (1994); 16S rRNA, primers from Palumbi (1994); nuclear histone 3 (H3), primers from Colgan *et al.* (2000); partial 28S, primers LSU5' (Littlewood *et al.*, 2000) and LSU1600R (Williams *et al.*, 2003). PCR was performed in 25 ml of reaction volume containing 22 ml of water, 0.5 ml of a forward and reverse PCR primer (10 pm/µl), 2 ml of template DNA solution and one puReTaq Ready-To-Go PCR Bead (GE Healthcare). The cycling parameters for amplification of COI, 16S and H3 consisted of an initial denaturation for 5 min at 94°C; followed by 36 cycles of denaturation for 45 s at 94°C, annealing for 50 s at 48°C, and

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extension for 200s at 72°C; and ended with a 10 min extension at 72°C. For 28S, amplification was performed with an initial denaturation for 3 min at 95°C; followed by 36 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 56°C, and extension for 120s at 72°C; and ended with a 10 min extension at 72°C. Successful PCR products were purified using the NucleoSpin Extract II (Macherey-Nagel GmbH & Co). Cycle sequencing using Big Dye 3.1 and the PCR primers (2 pm/µl) were conducted in the Genomic Service Unit of the Department of Biology, Ludwig-Maximilians-University Munich, Germany.

2.3 Sequence alignment and phylogenetic analyses

Sequences were edited using MEGA5 (Tamura *et al.*, 2011) and consensus sequences were generated in BioEdit (Hall, 1999). Some sequences were edited and consensus generated in Geneious R6 (6.1.5 version) (http://www.geneious.com, Kearse *et al.*, 2012). Nuclear H3 and partial 28S were identical among different samples (see Table 1), therefore these markers were not used for the phylogenetic and subsequent species delimitation analysis. For the phylogenetic analyses were used only specimens of which sequences of the two informative markers (COI and 16S) were available, i.e. individual and concatenated gene trees contain the same specimens and the same number of specimens. Alignments were generated with Muscle (Edgar, 2004) using the default settings. Testing the evolutionary models was carried out with Modeltest version 3.7 (Posada & Crandall, 1998). Substitution saturation rate of COI and 16S were measured with Xia's method implemented in DAMBE version 5.2.31 (Xia & Xie, 2001) for combined first and second codon positions, and for third codon position separately, using proportion of variation sites value of the best model obtained from Modeltest. The aligned 16S data set was masked with Aliscore (Misof & Misof 2009) which identifies ambiguously aligned regions in multiple sequence alignments. Single gene data sets were concatenated automatically using FASconCAT version 1.0 (Kück & Meusemann, 2010). Maximum likelihood (ML) single-gene and genes trees of the concatenated dataset were generated using RAxML v. 7.2.6 (Stamatakis, 2006) and node support was assessed with non-parametric bootstrapping with 1000 replicates. ML trees were visualized in FigTree v1.2 (http://tree.bio.ed.ac.uk/software/figtree/) and edited for publication in Corel Photo-Paint X6.

2.4 Species delimitation and haplotype network analyses

Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012), General Mixed Yule Coalescent model (GMYC) (Monaghan *et al.* 2009; Pons *et al.* 2006) and Poisson Tree Processes (PTP) (Zhang *et al.* 2013) were used as species delimitation analyses, using the same specimens and sequences as used for the phylogenetic analysis. ABGD is independent from predefined species entities and was applied using both COI and 16S data sets, using default values as also a higher limit for intraspecific divergence (Pmax = 0.3) in different models of evolution (Jukes-Cantor, JC69 or Kimura, K80). GMYC is a likelihood method for delimiting species by fitting within and between

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species branching models to reconstructed gene trees (Pons *et al.* 2006, Monaghan *et al.* 2009, Fujisawa & Barraclough, 2013). Ultrametric starting trees for GMYC were generated using BEAST 1.5.3 (Drummond *et al.* 2006, Drummond & Rambaut, 2007) from COI and masked 16S alignments, as described by Jörger *et al.* (2012). GMYC was performed in R using the SPLITS package (http://r-forge.r-project.org/projects/splits/) and analyses allowing single and multiple thresholds (Monaghan *et al.*, 2009) were performed. PTP model basic assumption is that the number of substitutions between species is significantly higher than the number of substitutions within species and this is reflected by tree branch lengths (Zhang *et al.* 2013), but it does not require the use of an ultrametric tree. PTP analyses were run with COI and 16S trees resulted from ML analyses and uploaded, individually, as nexus files in PTP webserver (http://species.h-its.org/ptp/) (Zhang *et al.* 2013). Trees were rooted and *Felimare kempfi* (MZSP 97623) was included as outgroup, as done in the phylogenetic ML analyses. PTP graphic results for each gene are presented as PhyloMaps (Zhang *et al.* 2011). Minimum and maximum pairwise uncorrected p-distances of COI, between and within the main clades were calculated with Species Identifier (Meier *et al.*, 2006). Haplotype networks for COI were constructed using statistical parsimony (Templeton *et al.*, 1992) implemented in the program TCS v1.21 (Clement *et al.*, 2000) with a connection limit of 95%. For the haplotype networks, all available sequences of COI were utilized. Diagnostic characters for COI and 16S, including homogeneous and heterogeneous characters (see Jörger & Schrödl, 2013), were obtained through Character Attribute Organization System (CAOS) software (Sarkar *et al.*, 2002; Sarkar *et al.*, 2008; Bergmann *et al.*, 2009) and cross-checked by eye, following the procedure described by Jörger & Schrödl (2014). In both cases we used the nucleotide data alignments applied for the phylogenetic analyses. Position numbers of diagnostic characters refer to the position in the alignment, that can be checked in the data matrices deposited in TreeBASE (www.treebase.org).

2.5 Reproductive morphology

In order to compare the results of the molecular phylogenetic and species delimitation analyses with the morphology, specimens belonging to the different groups (see Table 1) were dissected under a stereomicroscope, with special attention to the morphology of the reproductive system.

3. Results

3.1 Phylogenetic analysis and trees topology

The saturation analyses showed insignificant levels of saturation, even when the third codon positions were analysed independently. ML trees from single (COI, 657bp; 16S, 455bp) and combined (COI+16S, 1111bp) data-sets support *Felimida clenchi* complex as a monophyletic group (clade bootstrap support, bs = 99 for COI; 92 for 16S, and 100 for COI+16S), sister to a clade with *Felimida norrisi* and *Felimida sphoni* (Figure 2). The similar colored *F. krohni* clusters outside to the *F. clenchi*

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complex, *F. norrisi* and *F. sphoni* clade. In ML single gene trees (COI; 16S) and concatenated genes tree (COI+16S), *Felimida clenchi* complex is divided into three main clades. The distribution of specimens in the three main clades was identical in COI, 16S and concatenated trees (Figure 3). Nominal morpho-species, F. *clenchi*, *F. neona*, *F. binza* and *F. britoi,* were not recovered monophyletic. Morphotypes considered to be part of a certain species are distributed in different, well-supported clades. Specimens with color pattern traditionally assigned to *F. clenchi*, for example, are present in the three main clades of the ML trees. For COI, a clade (bs = 100) with Caribbean and Brazilian specimens of *F. binza*, *F. clenchi* and *F. neona* (Group A) is sister to a pair of clades, one (bs = 99) with Caribbean and St. Helena *F. clenchi* specimens plus Azorean, Madeira and Mediterranean specimens of *F. britoi* (Group B) which is sister to a small clade (bs = 96) with two '*F. clenchi*' specimens from Brazil (MZSP 97534 and MZSP 97601) (Group C) (Figure 3A). Concatenated analyses result in the same topology to the single COI tree, but with bs = 100 to each of the three main clades (Figure 3C). In the 16S tree, the clade with two Brazilian *F. clenchi* specimens (bs = 100) (Group C) is sister to the clade with Brazilian and Caribbean *F. binza* and *F. clenchi* specimens (bs = 64) (Group A), and not to the larger clade with *F. britoi* specimens (bs = 87) (Group B) as observed in COI and COI+16S trees (Figure 3B).

3.2 Genetic distances and species delimitation analysis

Concerning uncorrected p-distances for COI, Group A and Group B diverge in a minimum of 10.35%, Group A and B in a minimum of 9.58%, Group B and Group C in a minimum of 7.0%. Maximum divergence was of 1.97% within Group A; 2.89% within Group B, and of 0.6% between the two specimens of Group C. Analyses of COI data-set using default ABGD website values (Pmin = 0.001; Pmax = 0.1) and also a higher limit for intraspecific divergence (Pmax = 0.3) resulted mostly in three distinct species which correspond to the three main clades (Groups A, B and C) observed in the maximum likelihood phylogenetic analysis trees. These results were independent from the models of evolution selected (Jukes-Cantor, JC69 or Kimura, K80), with the same result also for simple distance. However, at lower values of prior intraspecific distance (P), recursive partition of ABGD recognized four species, separating the clade composed by specimens MNCN 47532, MZUCR 8431 and CPIC 528 from the rest of the Group A (Figure 4, parameter B). Analysis of 16S data-set using default ABDG website values resulted in three species which correspond to the clades of the tree from the ML analysis (Figure 4, parameter E). An exception was observed for lower P values using JC69 Jukes-Cantor distance which resulted in four species in the recursive partition, separating the clade with CNMO 3009, the GenBank JQ727708, three MZUCR specimens and the two Saint Helena specimens (ZSM 20130974 and 20130975) from the remaining Group B specimens; and resulted in five species in the initial partition, separating also the clade composed by specimens MNCN 47532, MZUCR 8431 and CPIC 528 from the rest of the Group A. GMYC single thresholds analysis for COI

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pointed for six different species, maintaining Group A and Group C as independent lineages but splitting Group B in four different species (Figure 4, parameter D). GMYC multiple thresholds analysis for COI resulted in an additional division, separating the specimen CNMO 3009 as an additional, seventh species. GMYC single thresholds analysis for 16S resulted in five different species, maintaining Group C as an independent putative species but splitting group A and group B in two different species each (Figure 4, parameter E). GMYC multiple thresholds for 16S pointed for six different lineages, with one additional division in Group A specimens. PTP analysis for COI and 16S resulted in three species, corresponding to groups A, B and C with high support in PTP Maximum likelihood solution for COI (Group A= 0.935; Group B= 0.988; Group C= 0.988) and good but less support for 16S (Group A= 0.941; Group B= 0.871; Group C=0.501) (Figures 4F and 5).

3.3 Haplotype networks

COI haplotype network analyses in TCS resulted in independent parsimony networks for each of the three main groups (Figure 6). Group A is distributed in two major groups of haplotypes, one containing only Brazilian and Costa Rica specimens and another with Virgin Islands, Cuba and Costa Rica specimens. In Group A, specimens with different color patterns from a same locality (Cabo Frio, specimens 16 and 19) or from distant points (Costa Rica and Virgin Islands, specimens 2 and 3) share a same haplotype. For Group B, no haplotype is shared between the 19 specimens. Group B network is distributed in four major groups: one with Costa Rica, Panama and one Mexican specimen, all with *F. clenchi* color pattern; a second group with Azores and Madeira specimens, all with *F. britoi* pattern; a third group with Azores, Madeira, Mediterranean (*F. britoi* pattern) and two Caribbean specimens (*F. clenchi* pattern); and a fourth group with the two specimens from St. Helena, both with *F. clenchi* pattern (Figure 4). The last haplotype network comprised the two specimens of the Group C, both from Cabo Frio, southeastern Brazil, with three substitutions between them.

3.4 Geographic distribution and sympatry

The three main groups, delimitated from the phylogenetic, ABGD, PTP and TCS analyses, have a partial overlap in their geographic distributions. Group A and Group B are sympatric in the Caribbean of Costa Rica where specimens with the same general color pattern are syntopic in localities such as Punta Mona (specimens 13 and 25, see Figure 8). Group A and Group C are syntopic in southeastern Brazil, Cabo Frio region, but the specimens presenting different color pattern (specimens 17 and 22, see Figure 8).

3.5 Nucleotide diagnostic characters

Groups A, B and C differ in 5 diagnostic characters for COI, with three single pure characters (positions 240, 270 and 522) and two single heterogeneous characters (positions 120 and 399) (Table 2). For 16S only a single pure character is present (position 284). Considering the differences only between sympatric groups, the Groups A and B, sympatric in Costa Rica, differ on 46 positions in COI

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and 14 positions in 16S (single pure characters only) (see Table 2). The Groups A and C, sympatric in Cabo Frio, differ in 57 positions in COI and 15 positions in 16S (single pure characters only) (Table 2). Position numbers refer to the positions in the matrix deposited in TreeBASE (link).

3.6 Reproductive morphology

No diagnostic differences were observed in the reproductive morphology between specimens of the groups A, B and C. The general disposition, shape and proportion of structures are similar, with variation in the length of the penial portion of the deferent duct and in the shape and size of the seminal receptacle among different specimens of group A and B. The single dissected specimen of group C presented a thinner seminal receptacle, but this cannot be considered informative without examination of further specimens.

4. Discussion

4.1 The Felimida clenchi *complex under molecular evidences*

Our molecular analyses support the monophyly of the *Felimida clenchi* complex group. With representatives from the Atlantic Ocean and the Mediterranean Sea, the *Felimida clenchi* complex is sister to a clade with two species from the tropical Eastern Pacific region: *F. sphoni* and *F. norrisi*. This corroborates the sister relationship between *F. clenchi* and *F. sphoni*, and *F. binza* and *F. sphoni* indicated in broader phylogenetic studies (Johnson & Gosliner, 2012, Ortigosa *et al.*, 2014). *Felimida krohni*, very similar in color pattern to *F. britoi*, and distributed in the same geographic region, clustered outside to the complex. However, our results on the molecular species delimitation of the complex are incompatible to all previous, morphology and color based taxonomic hypotheses. The separation into four species, each with a determined color pattern (Figure 1) (Ortea *et al.*, 1994) was not recovered in any of our analyses.

4.2 How many species in the Felimida clenchi *complex?*

We have a range of evidences, from mitochondrial and nuclear genes to color pattern, reproductive morphology and geographic distribution, to evaluate how many species are present in the *F. clenchi* complex. Also to confirm, or reject, our "different can be the same" and "the same can be different" color related hypotheses. Phylogenetic gene trees of two mitochondrial genes (COI and 16S) indicate three reciprocally monophyletic clades (Groups A, B and C) with a reasonable divergence when compared to inner divergence values. Species delimitation methods based on the barcoding gap, without predefinition or input of a gene tree data (ABGD), and also a branch length dependent method (PTP), reinforce the existence of three distinct species. GMYC splitted the three main groups in additional subdivisions, but this is somehow expected due to the sensitivity of the method, which is more responsive to effective population sampling, overestimating the number of species (e.g., Hamilton *et al.* 2014, Padadopoulou *et al.* 2009, Mirales & Vences, 2013). The high number of

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haplotypes distributed in smaller groups in Group B potentially favored the overestimating behavior of GMYC in our case.

The evidences from nuclear genes and the reproductive morphology, however, do not support the hypothesis of three species, resulted from the analyses based on the mitochondrial data set. No differences were observed on H3 and partial 28S sequences between specimens of Group A, B and C, including specimens from a same geographic area, as Costa Rica, as also from distant regions, such as Greece and St. Helena Island (Table 1). This different information obtained from mitochondrial markers in comparison to the nuclear markers plus reproductive morphology illustrates how species delimitation depends on the evaluation of the evidences and their relevance. Case by case it is up to the researcher to evaluate the special merits and limitations of data sets and delimitation methods, and integrate available evidences for a decision, having in mind that further, future evidences may challenge current opinions.

Nuclear loci, such as the H3 and the region of 28S sequenced in the present study, are generally more conservative than mitochondrial COI and 16S (REF), with examples in molluscs (Jörger *et al.* 2012, Malaquias *et al.* 2009) and particularly in nudibranchs (Ortigosa *et al.*, 2014, Pola *et al.* 2014). Previous studies showed little amount, or absence, of information in these loci to diagnose different sea slugs species. Just one nucleotide difference occurs in partial 28S between the two closed related, allopatric, *Bulla striata* and *Bulla occidentalis* (Malaquias *et al.* 2009). Two not directly related brightly colored nudibranch species of *Tambja* present only two differences in H3 sequences (*Tambja victoria* and *Tambja olivaria*; Pola *et al.* 2014) and no differences were found between H3 sequences of closed related species of *Spurilla* (Carmona *et al.* 2014) and *Felimare* (D. Ortigosa, pers. com). Overall, nuclear H3 and regions of the 28S seem to be very little informative, or not informative at all, for the delimitation of some nudibranch species. Similarly, the absence of a clear mechanical reproductive isolation observed in the *F. clenchi* group does not eliminate the possibility of other prezygotic barriers. Sexual behavior, chemical or genetic gamete incompatibility also can result in prezygotic isolation (Palumbi, 2009). And even if successful mating and fertilization occurs, the development can be unviable by postzygotic barriers (Kao *et al.* 2014).

In particular, the ambiguous evidences from color patterns are interesting. Although Groups A, B and C share specimens with the same or similar general pattern, they do not share identical morphotypes (Figure 8). The most similar specimens between the three groups, more specifically the 'dorsally circular spotted' form, present small but fixed differences, more clearly observable in the submarginal region of the mantle. Specimens 13 (Group A), 25 (Group B) and 22 (Group C) (see Figure 8) have a similar pattern, but are clearly different. The circular spotted form of group A (Figure 7, specimen 13) do not present the thin, transversal, red or dark purple dashes on the submarginal region. These dashes are present in the circular spotted forms of the group B (Figure 7, specimens 25,

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27, Mx and 43). Specimens of group C, in comparison, present a translucent grey submarginal band with light blue dashes and purple dots (Figure 7, specimens 22-23). The absence of shared identical morphotypes between the three groups is another factor that strengthens the hypothesis of three different, potentially mimetic, species, as pointed by the two mitochondrial markers.

The different lines of evidence thus guide towards different conclusions on the number of species in the *Felimida clenchi* complex. However, evidences are not units with a same weight and need to be evaluated in comparison to others. We resume it in an 'Evidence Power Line' (Figure 7). This line illustrates the evidences which strengthen (white arrows) or weaken (black arrows) each of the three main hypotheses of the number of species (four, one or three) in the *F. clenchi* complex. It can be seen also as a path to the taxonomic decision. According to the information available, discussed before, we consider more plausible and stronger the evidences that support the existence of three species within the complex (Figure 7). The fact that Groups A, B and C, delimitated through analyses of the molecular mitochondrial data, which also do not share identical morphotypes, occur in sympatry, with observed syntopy, enhances the three species hypothesis due to a presumptive evidence for their reproductive isolation (Jörger *et al.* 2014, Kekkonen & Hebert, 2014). Based on the available information the *Felimida clenchi* complex thus comprises three species, two of them polychromatic (Groups A and B). This supports our initial "different can be the same" hypothesis on species coloration and also the "the same can be different" hypothesis of specimens with the same general pattern belonging to different species, although the specimens of the different groups are not identical, as commented before. It is noteworthy to say, however, that species delimitation on the *F. clenchi* complex may suffer changes with the inclusion of potentially unsampled lineages and additional genetic markers. With further information, misleading aspects may emerge, such as incidents of introgression and incomplete lineage sorting, which could not be reliably evaluated with the present data (Figure 7).

4.2 Correlating delimitated species to names

A comprehensive integrative taxonomy approach should not only delimitate species but associate them to recognizable and available taxon names and, if not possible, propose new names in formal new species description (Jörger *et al.* 2014). We were unable to obtain sequences from type-specimens of the nominal *F. clenchi*, *F. neona*, *F. binza* and *F. britoi*, so we cannot associate them directly to any of our three delimitated species. With the absence of diagnostic differences from internal morphology, the association to existing names remains restricted to comparison to the original descriptions, original illustrations and the geographic distribution. The older name available is of *Felimida clenchi* (Russel, 1935), originally described from Bermuda, a dorsally red and yellowish pigmented species presenting a pair of anterior elliptic, elongated opaque areas that surrounds the base of each rhinophore and with a broad opaque-white submarginal band (Russel, 1935:59) (Figure 9A). Specimens like the Costa Rican

MZUCR8431 (Figure 8, number 3), from Group A, fits to the description of Russel (1935). Some other specimens of this group show variation in the amount of red and yellow pigmentation. The second name available is of *Felimida neona* (Marcus, 1955), originally as a species without the pattern of dorsal elongated or circular opaque-white and yellow spots, and with the presence of fluorescent red lines on the mantle, as observed in the topotype specimen MZSP 103241 (Figure 8, number 10), also part of the Group A. We thus conclude that Group A corresponds to *Felimida clenchi* (Russel, 1935) rendering *Felimida neona* (Marcus, 1955) a junior synonym; recovering the synonymy initially proposed by Meyer (1977) and followed by Thompson (1980) and Edmund & Just (1985).

The next name available is of *Felimida binza* (Marcus & Marcus, 1963), originally described from Curaçao, as having circular blue spots on dorsal mantle and transversal thin dark dashes on the submarginal band (Figure 9B). Some specimens of our group B, such as the Costa Rican and Mexican specimens (Figure 7, specimens 25, 27, Mx) fit to this original description. They have the same pattern, including the conspicuous transversal thin dashes on the submarginal band. The last name available is of *Felimida britoi* (Ortea & Pérez, 1983), originally described from the Canary Islands as a purple species with dorsal yellow or white lines, as can be observed in Madeira and Menorca specimens (Figure 7, specimens 37 and 42), also part of group B. We thus conclude that Group B corresponds to *Felimida binza* (Marcus & Marcus, 1963) and *Felimida britoi* (Ortea & Pérez, 1983) is herein considered its junior synonym. It is important to note that the current 'most in use' taxonomy on this group of species derived largely from the morphological revision of Ortea *et al.* (1994). In our opinion, and as commented before, in this revision the authors have attributed inverse concepts on *F. clenchi* and *F. binza,* if compared to these species original descriptions. Since then, erroneously and inversely, *F. clenchi* is being identified as the circular spotted form, with thin dashes on the submarginal region, and *F. binza* as the form with opaque white-yellow elongated spots and a white submarginal area (see Rudman 2000a,b). We herein reverse these taxon concepts back according to the species original descriptions. For Group C no existing name is available, so this species will be formally described in a separate publication.

4.2 Polychromatism and biogeography

Felimida clenchi and *Felimida binza* are polychromatic species but differ in the geographic distribution of their phenotypic variation (Figure 9). *Felimida clenchi* variation can be observed in a same geographic region or locality, including its extreme variation without yellow pigmentation (originally described as *F. neona*) (Figure 8, specimens 10 and 16), as also its spotted form (Figure 8, specimen 13), occurring together with the most common pattern (Figure 8, specimen 17) in southeastern Brazil and the Caribbean Sea, respectively. On the other hand, *F. binza* present different patterns according to its geographic distribution. The Eastern Atlantic and Mediterranean specimens are purple with yellow and white spots and lines and the Caribbean form resembles *F. clenchi* but

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always presenting dorsal circular spots and the submarginal region with thin transversal lines (Figures 8 and 9). Azorean specimens can be identical to other purple Eastern Atlantic and Mediterranean specimens or vary to light blue with yellow or orange lines and spots (Figure 8, specimen 29). St. Helena presents a different yellowish morphotype, with dorsal circular spots in a red and opaque yellow network, and submarginal purple spots. The color variation in *F. clenchi* is in general more randomly, while in *F. binza* seems to be a result of local selective pressure favoring a certain pattern of color. All but one, rare, species of *Felimida* from the eastern Atlantic and Mediterranean Sea are also purple with yellow or white spots (Figure 9). We believe that the repetitive occurrence of this color form in different, including not closely related (Ortigosa *et al.* 2014) eastern Atlantic *Felimida* species, results from a local evolutive pressure to its maintenance, as hypothesized by Ros (1976). In the tropical Western Atlantic the *Felimida* diversity is lower and the other few congeneric species, such as *F. grahami* and *F. regalis* in the Caribbean Sea, and *F. paulomarcioi* in Brazil, present a whitish mantle with faded salmon to orange spots (Domínguez *et al.* 2006, Valdés et al, 2006, Camacho-García *et al.*, 2014) (Figure 9). Together with *F. clenchi*, *Felimida binza* is the most common and widespread *Felimida* species in the Caribbean region and their similarity in color may result from local, probably reciprocal, pressure resulting in mimetic patterns. This would explain the existence of unusual circular spotted forms of *F. clenchi* (Figure 8, specimen 13), inclusive in syntopy to *F. binza* specimens. The hypothesis of a local selective pressure, as predators able to recognize and remember color patterns, which results in the different geographic color patterns of *F. binza* is strengthened by the fact that some Azorean specimens and the St. Helena specimens of do not resemble other *Felimida* species. Both Azores, with the co-occurrence of just *F. purpurea*, and St. Helena, with no other known *Felimida* species, would represent regions with less pressure directed to 'imitate' a certain color pattern.

For the first time, molecular data supports the existence of extreme color polymorphism in brightly colored nudibranch species. This has direct implication to the traditional taxonomy of the group and its biodiversity, as species identification based on differences in color pattern potentially have taken to wrong conclusions. In addition to polychromatism, the three species part of the *Felimida clenchi* complex share similar morphotypes. Works on terrestrial chromatic species, such as butterflies of the genus *Heliconius*, showed the existence of many cases of polychromatic species and Müllerian mimicry (The Heliconius Genome Consortium, 2012). Although initially commented (Ros, 1976, Edmunds, 1981, 1987) and discussed years ago (Gosliner & Behrens, 1990, Rudman, 1991), the existence and origin of mimicry and mimetic color groups in nudibranchs remain unexplored. Polychromatism and mimicry in *Heliconius* butterflies result mostly from hybrid speciation and adaptive introgression, favoring genes regulating the aposematic wing patterns (Heliconius Genome Consortium 2012; Pardo-Díaz *et al.* 2012; Kozak *et al.* in press), including the existence of mimicry

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supergenes (Joron *et al.* 2011). Similar mechanisms were observed in other chromatic groups, as damselflies, cichlid fish and birds (Sánchez-Guillén *et al.* 2005, Wellenreuther *et al.* 2014). Based on the evidences from terrestrial and fresh water chromatic groups of animals, it seems reasonable to think that similar events occur along the evolution of nudibranchs and other marine groups. Further studies are necessary to test our preliminary hypothesis on the origin of polychromatism and similar color patterns observed in species of the *Felimida clenchi* complex.

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Figure Captions

Fig. 1. Color pattern of the four species of the *Felimida clenchi* complex, following Ortea *et al.* (1994). (A) *Felimida clenchi*, (B) *Felimida neona*, (C) *Felimida binza* and, (D) *Felimida britoi*.

Fig. 2. Maximum Likelihood inferred COI+16S concatenated genes tree, including 36 specimens of the *F. clenchi* complex. Tree rooted using *Felimare kempfi* as outgroup. Bootstrap support values are shown above branches.

Fig. 3. Topology of the *Felimida clenchi* complex clade resulting from the Maximum Likelihood analyses. (A) COI gene tree, (B) 16S gene tree, (C) Concatenated COI+16S genes tree. Bootstrap support values are shown above branches. The three main groups resulted from the different analyses labeled as Group A, Group B and Group C in the concatenated tree.

Fig. 4. Color morphs, geographic distribution, TCS analysis and the different species delimitation methods results plotted in the ML concatenated COI+16S genes tree topology of the *F. clenchi complex*. I. Traditional species color morphs: red = *F. binza*, yellow = *F. clenchi*, green = *F. neona* and blue = *F. britoi*. II. Geographic distribution: CB = Caribbean Sea, BR = Brazil, EM = Eastern Atlantic/Mediterranean Sea, SH = Saint Helena Island. A. TCS (connection limit of 95%), independent parsimony haplotype networks. B. ABGD, based on COI data-set. C. ABGD, based on 16S data-set. D. GMYC, single thresholds for COI. E. GMYC, single thresholds for 16S. F. PTP result based on both COI and 16S data-sets. Specimens illustrated belonging to one of the three main groups resulted from the phylogenetic and species delimitation analyses. Specimen numbers refer to Table 1 and Figure 6.

Fig. 5. PhyloMap-Poisson Tree Processes (PTP) based on COI data-set. Branch connecting *F. krohni* only partially represented.

Fig. 6. TCS (connection limit of 95%) haplotype networks for COI gene. Each circle represents a haplotype, lines between nodes represent a single base change, and the size of the circle represents haplotype frequency. Color in the circles are referent to the color pattern of each specimen, according to the traditional taxonomy (yellow= *F. clenchi*, green= *F. neona*, red= *F. binza*, blue= *F. britoi*). Specimen numbers refer to Table 1 and Figure 8.

Fig. 7. The Evidence Power Line (EPL), a path to the taxonomic decision. Evidences supporting (white arrows) or weakening (black arrows) the three main hypotheses of the number of species (in blue) in the *Felimida clenchi* complex.

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Fig. 8. Specimens of showing the color patterns and variation of *Felimida clenchi* (Group A, green), *Felimida binza* (Group B, purple) and *Felimida* sp. (Group C, blue). For specimens information see Table 1.

Fig. 9. Biogeography and species color of the *Felimida clenchi* complex. Green area: *Felimida clenchi*; Pink area: *Felimida binza* and; Blue area: *Felimida* sp. The species *Felimida clenchi* and *F. binza* are sympatric in the Caribbean Sea and *F. clenchi* and *F.* sp. are sympatric in southeastern Brazil. Smaller adjacent specimens represent the color pattern of other species congeneric present locally.

Optional Figure.

Original illustrations of *Felimida clenchi* and *Felimida binza*, respectively. Adapted from Russel (1935) and Marcus & Marcus (1963).

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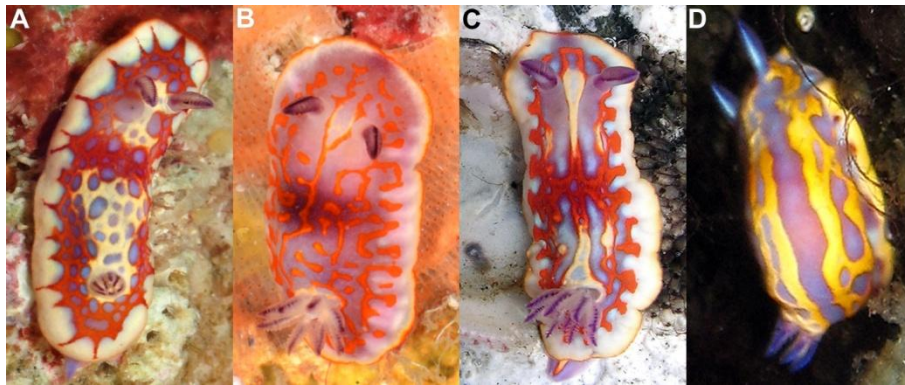
Figure 1

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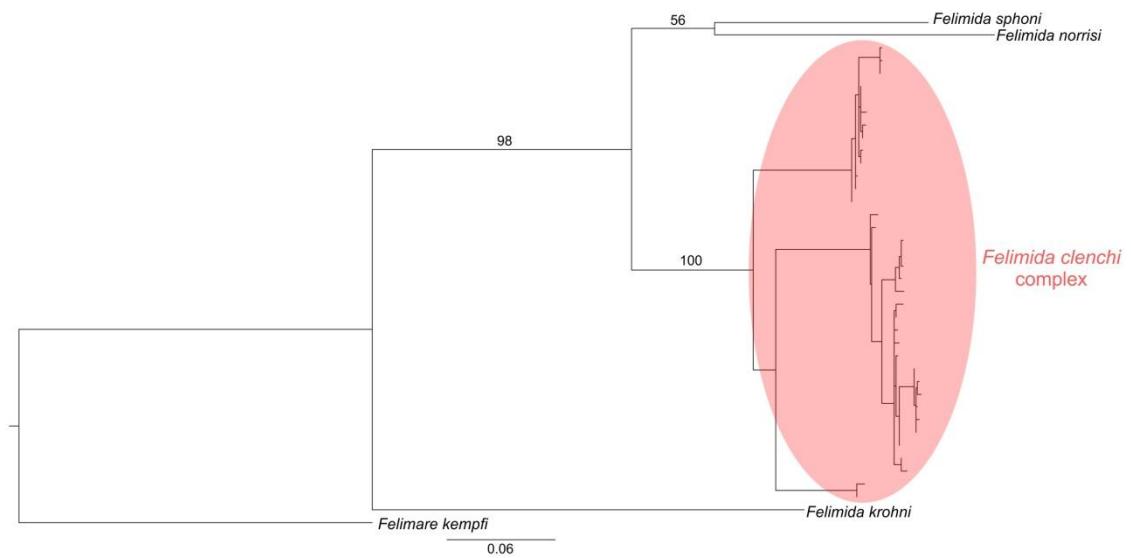
Figure 2

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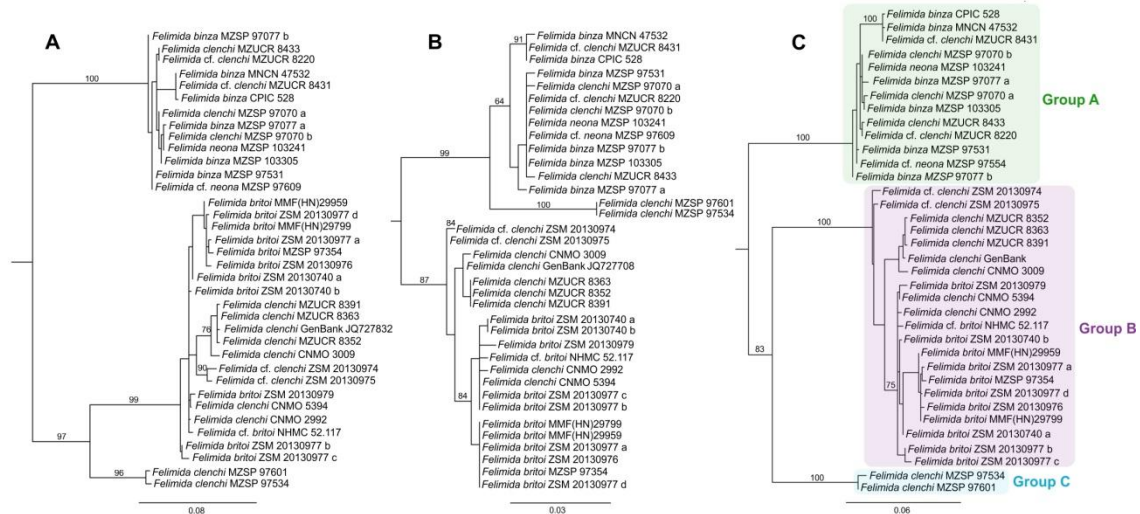
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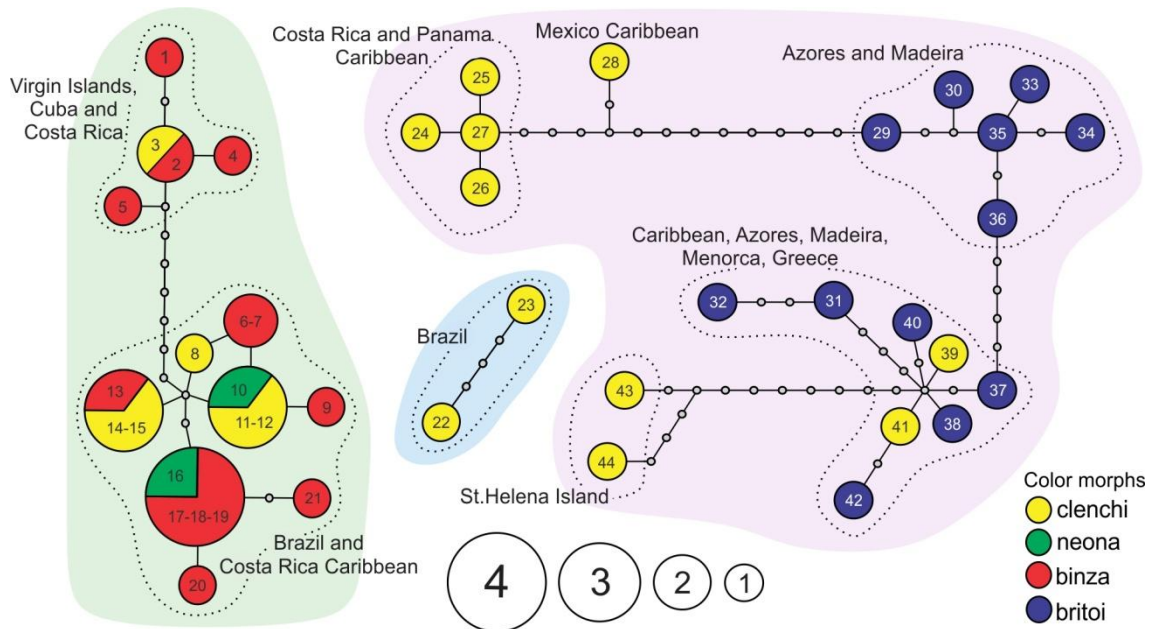
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Figure 7
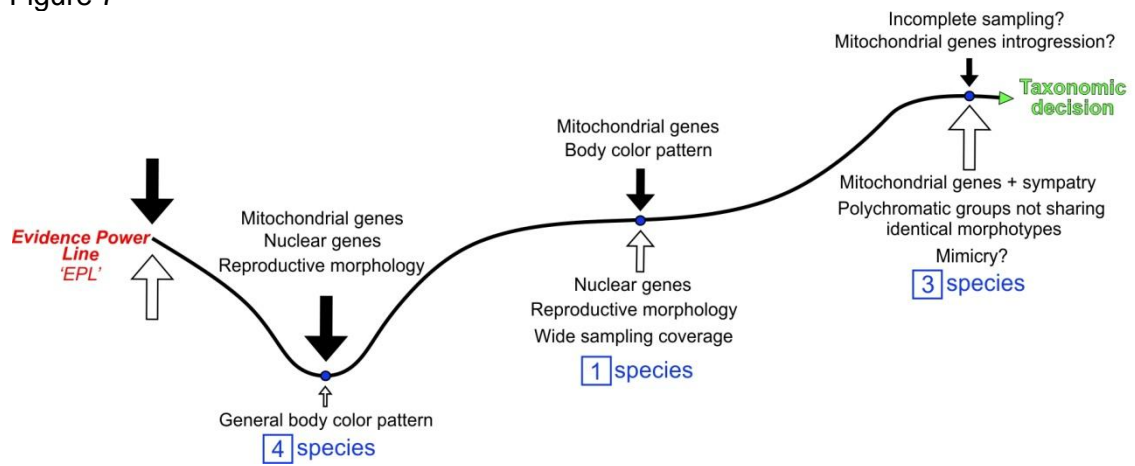
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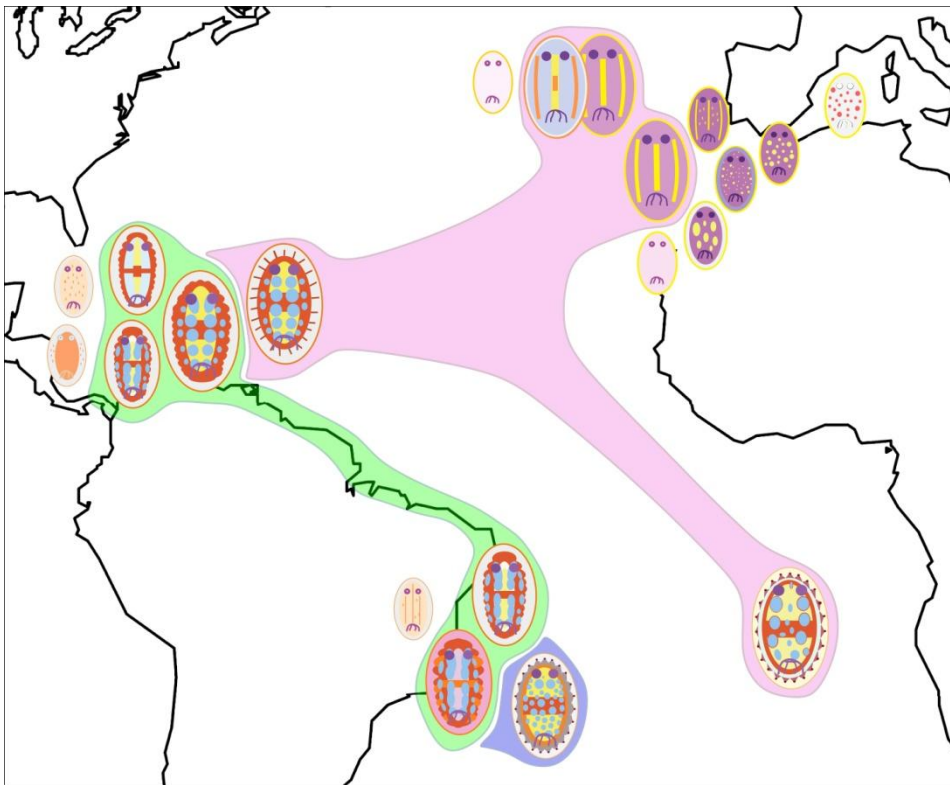
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Optional Figure
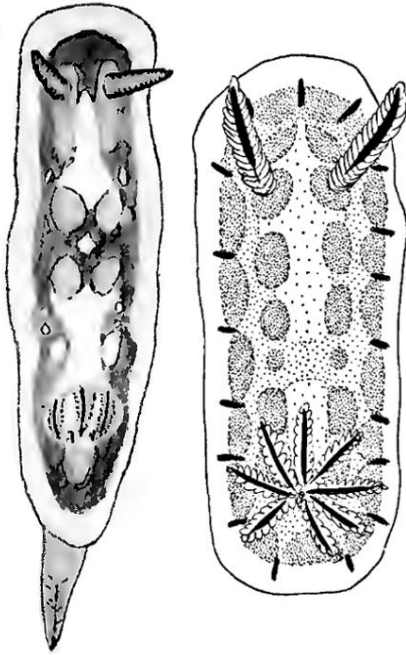
Optional Figure.
Original illustrations of *Felimida clenchi* and *Felimida binza*, respectively. Adapted from Russel (1935) and Marcus & Marcus (1963).

4. DISCUSSION

4.1 New data on Brazilian opisthobranch diversity

As expected, the surveys along southern and northeastern Brazil (Padula et al. 2011, 2012), in the Caribbean coast of Costa Rica (Camacho-García et al. 2014), and around Ascension Island (Padula et al. 2014b) revealed a previously unknown magnitude of opisthobranch biodiversity in these regions. This includes the discovery of species known from elsewhere, as well as undescribed species. In these four papers, some species are illustrated with color photos of living specimens for the first time, increasing chances for accurate re-identification in future works. The new records of the nudibranchs *Felimida paulomarcioi* and *Tambja stegosauriformis* from Santa Catarina, Brazil (Padula et al. 2011), expand their known geographic distribution more than 900 km southwards, entering in a subtropical region. As observed previously for other tropical western Atlantic species, as some reef fish (Barneche et al. 2009), the new records in subtropical waters of southern Brazil suggest that some species can tolerate different environmental and ecological conditions, as colder waters of Santa Catarina. Another possibility is that the occurrence of tropical species in southern Brazil results from occasional climatic events, such as temporary currents which could take larvae stock to the region, similarly to events observed in some regions of the coast of Peru (Uribe et al. 2013, Schrödl & Hooker 2014). The 28 new local records in Alagoas (Padula et al. 2012) comprise more than the total of 22 opisthobranch species that were previously known from the region (Marcus, 1971, Rios, 1994, García et al., 2008). Eleven of the new 28 records are also new records from the northeastern Brazilian coast. For most of the species, these records apparently fill a gap of their known geographic distribution between the Caribbean Sea and southeastern Brazil (Valdés et al., 2006). This is the case, for example, in *Elysia subornata* Verrill, 1901, *Felimida binza* (Marcus & Marcus, 1963) and *Flabellina engeli* Marcus & Marcus, 1968. Due to sparse data available from most regions of the Brazilian coast a recent hypothesis on the biogeography of Brazilian opisthobranchs (García et al. 2008) is herein shown to be not conclusive. For example, the biogeographical region of Alagoas state included only 30 species (García et al. 2008: 198). Padula et al. (2012) nearly doubles the number of species known from the region, with a high number of species more common to the Caribbean Sea than to southeastern Brazil. Thus, biogeographical approaches on Brazilian opisthobranchs are still dependent on more faunal surveys as the ones by Padula et al. (2011, 2012), which will result in necessary data on species richness in the different regions of the Brazilian coast.

The survey of the Caribbean coast of Costa Rica resulted in the collection of 70 species, of which 17 represent new records for the country, including undescribed species (Camacho-García et al. 2014). For most of these species, the records from the coast of Costa Rica represent important range extensions on their geographic distribution as they had never been previously found west of Jamaica, Cuba, or Colombia (Valdés et al. 2006). The new specimens, pictures and information on the species obtained by Camacho-García et al. (2014) provide data to be included in integrative systematics studies (see chapters 6-11), allowing, for example, a better understanding of the limits between intraspecific variation and interspecific differences. Among the 70 species found in the expedition to Costa Rica (Camacho-García et al. 2014), at least 40 are recorded also in Brazil (García et al., 2008). The similarity between the Caribbean and Brazilian opisthobranch fauna was initially observed and discussed by Ev. Marcus & Er. Marcus (1960) and Edmunds (1964). Dowgiallo (2004) mentioned that among 960 gastropod species occurring in the Bahamas, which is even northern to the Caribbean Sea, at least 480 (50%) occur in Brazil.

Some marine species initially recognized as having this wide geographic distribution pattern in the tropical Western Atlantic region were, with more detailed morphological and molecular studies, identified as distinct pairs of species, one occurring in the Caribbean and one in the Brazilian coast. This was observed in reef fish (Sazima et al. 1998, Rocha & Rosa 2001), with the Amazon outflow considered the main barrier to promote speciation events (Vermeij 1978, Rocha et al. 2001). On the other hand, molecular studies supported the occurrence of a same species over long distances in the western Atlantic, as is the case of the sponge *Chondrosia reniform* (Nardo, 1847) from Bermuda to southeastern Brazil (Lazoski et al. 2001), and the tunicate *Phallusia nigra* (Savigny, 1816) distributed from Florida to Panama and southeastern Brazil (Nóbrega et al. 2004). In the case of reef fish it has been suggested that the connection between the Caribbean and the Brazilian east coast, regions theoretically isolated by the Amazon River outflow, would occur through banks of sponges situated between 50 and 70 meters deep, where there is little sedimentation and higher salinity (Rocha, 2003). The records of (benthic) opisthobranchs in depths of 23-52 meters in an area still under influence of the Amazon outflow (Ev. Marcus, 1971) supports the hypothesis of connection between the Caribbean and Brazilian populations through deeper waters as discussed by Rocha (2003). However, the role of the Amazon outflow on usually pelagic opisthobranch larvae has yet to be explored.

Having studied several cases of potential opisthobranch species complexes during my doctorate, it appears that the distance and the Amazon River outflow between the Caribbean Sea and Brazil act more as a filter than as strict barrier for species distribution and speciation events in the Western Atlantic. In the case of *Navanax aenigmaticus* (see Ornelas-Gatdula et al. 2012), *Felimida clenchi* (see chapter 11) and *Discodoris branneri* (Figure 2), Caribbean and Brazilian specimens were confirmed as conspecific. The same was observed for the intersticial opisthobranch *Pontohedyle brasiliensis* (Rankin, 1979) by Jörger et al. (2012).
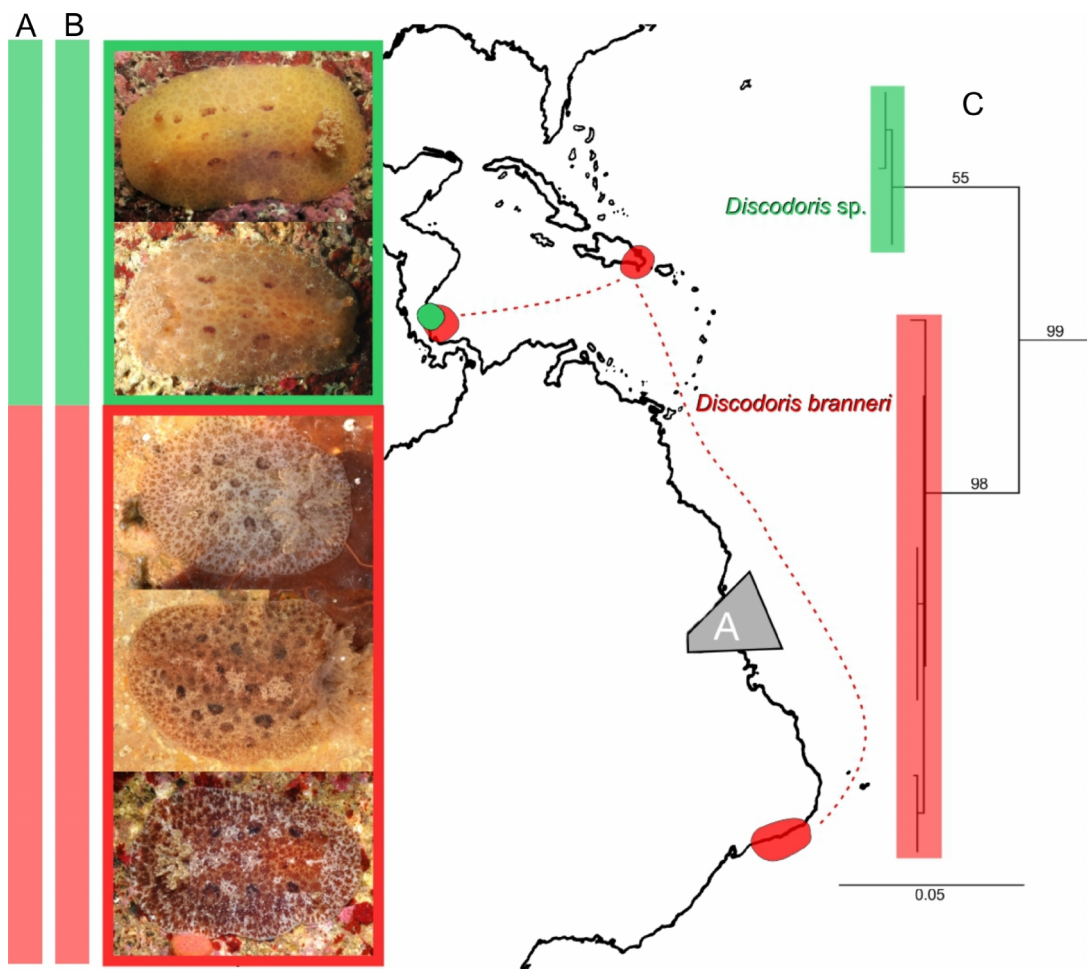
Figure 2. *Discodoris branneri* (demarcated in red) is widely distributed in the tropical Western Atlantic but has a sympatric cryptic species in Costa Rica (demarcated in green). **A**. Reproductive morphology. **B**. ABGD delimitation analysis based on COI alignment. **C**. Tree topology (only ingroup represented) resulted from Maximum Likelihood analysis of COI gene. Bootstrap support values are shown above branches. The grey area demarcated with a white 'A' represents the region with the Amazon River outflow.

On the other hand, Caribbean and Brazilian specimens identified as *Pleurobranchus areolatus* belong, in fact, to two different, not close related species (Goodheart et al. 2015) and the geographically separated cryptic species detected in the *Nanuca sebastiana* complex (Figure 3, Padula et al. in prep.) may have originated under the influence of the Amazon River outflow. Interestingly, an undescribed *Nanuca* from Atol das Rocas seems to be more closely related to the Caribbean species than to the ones from the Brazilian coast (Figure 3), requiring further biogeographic investigations.
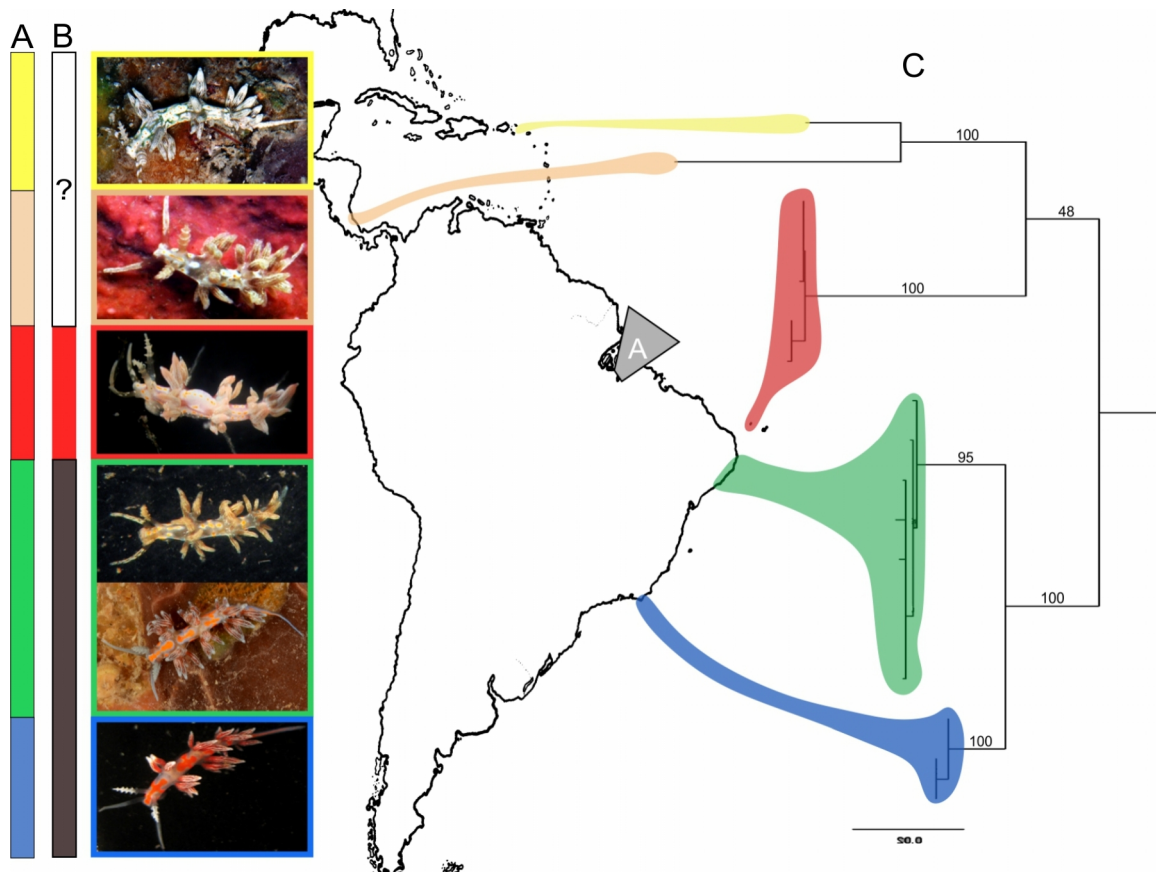
Figure 3. *Nanuca sebastiana* represents a complex of at least four species: one in the Caribbean (yellow and orange label); one at Atol das Rocas (red), one in northeastern (green) and another in the southeastern Brazilian coast (blue). Caribbean clades are represented by only one specimen each, restricting further conclusions. **A**. ABGD and PTP delimitation based on COI data-set. **B**. Reproductive morphology (absence of data from the Caribbean representatives). **C**. Tree topology (only ingroup represented) resulted from Maximum Likelihood analysis of concatenated COI+partial 28S genes data-set. Bootstrap support values are shown above branches.

Our work on Ascension Island (Padula et al. 2014b) resulted in the addition of seven new records of species to this small mid-Atlantic island, from where eight opisthobranch species were previously reported (Smith 1890a,b, Rosewater 1975). Among the new findings were two new species (Padula et al. 2014b). In total, now 17 species are known from Ascension, six of them not restricted to the Atlantic Ocean, with records in the Pacific Ocean. However, as emphasized by Padula et al. (2014b), traditional taxonomy based on

few morphological characters was probably masking complexes of species. One example is *Pleurobranchus areolatus*, which was previously recorded from the eastern Pacific, Caribbean Sea, Brazil, Azores and Cape Verde. Our recent molecular phylogeny and species delimitation on *Pleurobranchus* (Goodheart et al. 2015) indicate that *P. areolatus* comprehends a complex with at least three species, one from the Eastern Pacific, one from the Caribbean Sea and the last occurring in Brazil, Azores and Cape Verde. For the last, the name *P. reticulatus* was recovered and this is the species recorded from Ascension (Goodheart et al. 2015). It is known that Ascension and St. Helena shallow water marine fauna receives influence from both western and eastern Atlantic coasts, including the occurrence of amphiatlantic reef fish species (Floeter et al. 2008), but, in fact, data is still scarce for many invertebrate groups. Briggs & Bowen (2012) compiled biogeographic data and reported the mass of water separating western Atlantic and eastern Atlantic as a soft barrier for dispersion. However, amphiatlantism was not confirmed for different opisthobranch species in recent studies (Carmona et al. 2011, Ornelas-Gatdula, 2012). Of the 17 species known from Ascension Island, 11 are found in Brazil. This supposedly similarity needs to be confirmed through integrative taxonomic approaches, using e.g. morphological and molecular evidences.

In all cases studied in the present thesis of species traditionatelly considered transpanamic or with tropical amphi-South America distribution, this geographic distribution pattern was rejected. This is the case, for example, of '*Navanax aenigmaticus*' which consist in fact of two distinc species in the eastern Pacific and western Atlantic, respectively (Ornelas-Gatdula et al. 2012), and '*Pleurobranchus areolatus*', which in fact corresponds to distinct, not close related species in the eastern Pacific, the Caribbean Sea and the Brazilian coast (Goodheart et al. 2015). However, the high genetic similarity between Atlantic and Pacific specimens of other sea slugs species, such as *Spurilla* species (Carmona et al. 2013) requires further investigations.

4.2 Computer-based 3D reconstruction of *Pluscula cuica*

The use of 3D-microanatomy reconstruction from serial semithin histological sections allowed the observation and obtainment of new and relevant histological and morphological information in the taxonomy and systematics of opisthobranchs (e.g. Neusser et al. 2006, 2009; Jörger et al. 2008, Rückert et al. 20082010b; Eder et al. 2011). In particular, this technique allowed detailed redescriptions of small species, such as interstitial acochlideans, sacoglossans and nudibranchs (Jörger et al. 2014), which could not

be studied in detail through manual dissection under an optical microscopy. The accumulation of new information on these groups and their inclusion in general phylogenetic studies changed the traditional concepts on the classification and systematics of the Heterobranchia (Jörger et al. 2010, Schrödl et al. 2011, Wägele et al. 2014). The Brazilian *Pluscula cuica* Marcus, 1953 was described presenting a thin internalized shell, the genital opening in a posterior position, still separate cerebral and pleural ganglia, and five distinguishable ganglia on the visceral nerve cord (Marcus, 1953), indicating a potential basal position among the philinoglossans. Marcus (1953) suggested that the mode of autosperm transfer would be through the hemocoel instead of being transported along the external ciliated groove along the right body side, as in most other cephalaspideans. Due to these peculiarities, some authors place *Pluscula cuica* in a family of its own, Plusculidae (Marcus 1959; Franc 1968; Bouchet and Rocroi 2005) or subfamily, Plusculinae (Salvini-Plawen 1973). Our 3D-microanatomy reconstruction revealed several "accessory" ganglia, a reduced posterior mantle cavity that retains some putative shell-building tissue, but the shell is absent, and an osphradium (Brenzinger et al. 2013b). Contrary to originally described, autosperm is transferred to the cephalic copulatory organ via an external sperm groove, not through the hemocoel, and the penis opens through the oral tube (Brenzinger et al. 2013b). The supposed primitiveness of the fused rather than separate cerebropleural ganglia and the triganglionate rather than pentaganglionate visceral nerve cord was based on misobservations. Thus, the higher categories, such as Philinoglossacea for Philinoglossidae for allocation of *P. cuica* were considered no longer warranted (Brenzinger et al. 2013b). Inner cephalaspidean relationships await further evidences from molecular studies. 3D-microanatomical examination thus was highly useful for assessing and correcting original description, and also provided substantial descriptive detail on organs and tissues not regularly used for taxonomic purposes. Such "deep descriptions" are potentially useful for characterizing and delimitating species, and usually the only option for microscopic species (Jörger et al. 2014). While promising to morphologically track recent or rapid radiations, revealing such features from a wide range of specimens and populations is highly time-consuming. For macroscopic species thus traditional dissecting techniques appear more efficient. However, their adequacy to delimitate separate opisthobranch evolutionary lineages has been seldomly tested.

4.3 Traditional taxonomy versus integrative taxonomy

When we found *Babakina festiva* and *Aeolidiella alba*, two putative Indo-Pacific nudibranch species, at the Brazilian coast, we suspected that these species rather could represent complexes of species with a more restricted geographic distribution (Padula et al. 2005, 2006). This was confirmed later in a taxonomic revision and as a secondary result of a broader phylogenetic study (Gosliner, Garcia-Duarte & Cervera 2007, Carmona et al. 2014). Thus, *Babakina festiva* and *Aeolidiella alba* in fact do not occur in Brazil; instead the Brazilian *Babakina* seems to be *B. anadoni*, a Mediterranean species (Carmona et al. 2011b) and Brazilian *A. alba* is an undescribed species of the genus *Bulbaeolidia* (Carmona et al. 2014). These are examples of two cases among many others which always intrigued me: the repetitive records of opisthobranch species with wide, unexpected geographic distribution. There are considerable numbers of opisthobranch species which are recorded as circumtropical, cosmopolitan, or with wide geographic occurrence, e.g. amphi-South American or occurring from the Red Sea to the Pacific Ocean (Schrödl 2003, García et al. 2008, Yonow 2015). These patterns of geographic distribution would not be expected according to the reproductive biology and ecology of most opisthobranch species. They have a benthic, slow moving, adult stage and a limited dispersal capacity as planktonic larvae (Goddard 2004). Even tough, until today, many specialists have not questioned the paradigm, i.e. whether the repetitive pattern of wide geographically distributed opisthobranch species is real or a result of limited scope of the traditional taxonomy.

In some marine groups, such as sponges and small mesopsammic sea slugs, taxonomy is generally difficult by the reduced number of informative morphological characters. Due to this fact, some authors decided to go further looking for additional evidences, such as molecular markers, to a better delimitation of species (e.g. Klautau et al. 1999, Jörger et al. 2012, 2014). In the case of larger, conspicuous opisthobranchs, such as the Nudibranchia, taxonomy is traditionally based on external morphology, body color, radula and reproductive system (Thompson 1976). In theory, the more diverse range and variability of characters would allow for a better delimitation of species. However, with the use of molecular markers in general phylogenetic studies (e.g. Pola et al. 2007, Malaquias & Reid 2008, Carmona et al. 2013) potential cryptic species started to be discovered. This cryptic diversity was detected also in the first studies focused on potential species complexes in opisthobranchs (e.g. Ornelas-Gatdula et al. 2012, Krug et al. 2013). Together with the wide, unexpected geographic distribution of some species, a series of other evidences strengthen doubts on the limits of the traditional taxonomy to delimitate

opisthobranch species. For example, I have observed specimens with different color morphs, which were traditionally identified as different species, occurring repeatedly in syntopy along the Brazilian coast. Furthermore, evidences emerged that sea slugs radulae are not as static and characteristic as previously thought: radulae can present a same shape in different species (Carmona et al. 2014) or are extremely variable within a species, as for example among adults of *Tyrinna nobilis* Bergh, 1898 (Schrödl & Millen 2001) or during the ontogeny of *Dendronotus* spp. (Ekimova et al. 2015) being not a reliable diagnostic character as traditionally believed. Based on these considerations, a series of conditions and patterns is suggestive that traditional taxonomy is potentially failing to delimitate opisthobranch species:

I. Species with wide geographic distribution or wide hydrographic tolerance,

        IA. recorded in different oceans or across barriers (or filters) in a same ocean;

        IB. incompatible or improbable considering its reproductive biology (e.g. short planktonic larvae period) and ecology (e.g. specialized, restricted diet).

II. Species presenting regionally different morphotypes (e.g. with different color patterns or body size)

III. Species, mostly in sympatry, with a same general body color pattern but presenting small consistent, apparently fixed differences.

IV. Taxonomic decisions based on limited sampling, not covering or not considering possible intraspecific variation of color pattern and other taxonomic characters

V. The proposition of new species names based on the observation of few differences in body color pattern or radula or reproductive system - such as proportion of allosperm receptacle, which can vary depending on reproductive activity - without any further supporting evidence.

        My integrative taxonomic research on the different cases studied along the doctorate (Table 1), in addition to the results of recent species delimitation works on opisthobranchs based on molecular data (Valdés et al. 2011, Ornelas-Gatdula, 2013, Goodheart & Valdés 2013, Krug et al. 2013), indicate that the traditional taxonomy on opisthobranchs is failing in its main objective: identify species and the limits between species.

Species case	Geographic Distribution	Number of species (Traditional taxonomy)	Number of species (Integrative Taxonomy)	Morphological differences	Color pattern differences	Remarks	Reference
Navanax aenigmaticus (Bergh, 1893)	Tropical Eastern Pacific, Western and Eastern Atlantic	one	three	yes internal shell morphology	no	three different species in allopatry	Chapter 6
Cratena peregrina (Gmelin, 1791)	Mediterranean Sea, Eastern Atlantic and Brasil (?)	one	two	yes Species length and reproductive morphology	yes	same general color pattern but with small fixed differences	Chapter 7
Pleurobranchus areolatus Mörch, 1863	Tropical Eastern Pacific, Western and Eastern Atlantic	one	three	yes In radula and reproductive morphology	no	polychromatic species	Chapter 9
Felimida clenchi complex	Tropical and subtropical Atlantic and Mediterranean Sea	four	three	no	yes	extreme polychromatism Similar patterns in syntopy, potential case of mimicry	Chapter 11
Nanuca sebastiani Marcus, 1957	Caribbean Sea and Brazil	one	four or five	partial penial morphology	in some species	one polychromatic species;.	Padula *et al.* in prep.
Discodoris branneri MacFarland, 1909	Caribbean Sea and Brazil	one	two	yes penial morphology	yes	*D. branneri* wide distributed; a cryptic species in Costa Rica.	Padula *et al.* in prep.
Mexichromis kempfi (Marcus, 1971)	Caribbean Sea and Brazil	one	two	no	yes	Caribbean and Brazilian species are different species	Padula *et al.* in prep.
Felimida grahami and *Felimida paulomarcioi*	One in the Caribbean Sea, another in Brazil	two	one	no	no	single species with variation in color pattern	Padula *et al.* in prep.
Aplysia parvula Mörch, 1863	Circumtropical	one	at least three	?	no	different species in different oceans	Valdés *et al.* in prep.

Table 1. The main different cases investigated through an integrative taxonomy approach in the present study.

Jörger et al. (2014) proposed an integrative taxonomic workflow designed for delimitating elusive, rare, mesopsammic sea slugs. These animals are mostly microscopic, larger specimens have just few millimeters of length, and there is a need of special techniques to extract them from sand grains (Schrödl, 2006). Most species are also translucent white and closely related species may occur in syntopy but look identical. Multi-locus barcoding is recommended for each single specimen obtained, and complex steps of congruence and compatibility considerations recommended to fully exploit any hidden signals (Jörger et al. 2014). On the contrary, larger opisthobranchs, such as the members of the Nudibranchia, present a diverse morphology and many species are also brightly colored. Thus, nudibranchs and other larger opisthobranchs may not require some of the laborious and expensive steps of the workflow proposed by Jörger et al. (2014). Due to the differences to mesopsammic and other elusive marine groups I propose a cost-effective integrative taxonomic workflow for larger, conspicuous opisthobranchs, which can be expanded for any other conspicuous marine, benthic, coastal shallow water groups (Figure 4). The first step on the taxon in question is checking main evidences, listed above,

of potential imprecise species delimitation through the traditional taxonomy, because they may suggest the need of further taxonomic investigations. Further steps of the herein proposed workflow are shown in Figure 4. Treating with non-abundant zoological groups, the approach requires a minimum of three specimens per locality or per morphotype, if available, and a minimum of sequencing a barcoding gene, which in general is the mitochondrial cytochrome oxidase I (COI) (Hebert et al. 2003a,b). However, for some groups COI cannot be easily obtained (e.g. polyclad flatworms; Litvaitis et al. 2010) or shows slow evolution rate (e.g. in Anthozoa; Shearer et al. 2002), thus the selection of another informative locus is necessary. In any case, a higher number of loci, preferentially independent, and specimens, would increase the support of molecular evidences. Adding further mitochondrial genes to a COI-barcode may be useful to increase support and resolution, but mitochondrial genes are linked. Therefore, the addition of an independent nuclear marker to a mitochondrial one is highly recommended. The alignments of the edited sequences of the different loci are the base for the phylogenetic and the species delimitation analyses and for detection of molecular diagnostic characters. Special care should be invested to make alignments as accurate, transparent as also accessible in a public database (see Jörger & Schrödl 2013). Diagnostic nucleotide characters represent a simple way to indicate the molecular differences between species, in addition to describable morphological, ecological and biological differences (Ornelas-Gatdula et al. 2012, Jörger & Schrödl 2013, Padula et al. 2014a). Hypotheses on species groups need to be compatible, i.e. not contradicted, but not necessarily recovered by all types of data and analyses. For example, some nuclear markers broadly used for delimitating opisthobranch species, i.e. H3 and 28S, can evolve too slowly to flag recent diversifications in some opisthobranch groups, such as some Chromodorididae (Padula et al. in prep.). However, different loci can also show incongruent information, and various analyses may result in contradicting evidences (Toews & Brelsford 2012). Rather than ignoring such results, or downweighting their relevance as disturbing artifacts, the possibility of different evolutionary rates of the genes as also potential events of introgression and incomplete lineage sorting must be considered. The last events have been recently observed in other groups, such as butterlies (The Heliconius Genome Consortium 2012) and lizards (McGuire et al. 2007). Thus, incongruent gene trees do not necessarily contradict certain species hypotheses, and species sensu de Queiroz (2007) do not necessarily result as monophyletic in different genes trees. However, this knowledge (and appropriate species tree approaches) still needs to be implemented as a general practice in opisthobranch taxonomy. The different species

delimitation approaches, e.g. Automatic Barcoding Gap Discovery (ABGD; Puillandre et al. 2012), and General Mixed Yule Coalescent model (GMYC; Pons et al. 2006, Monaghan et al. 2009) have different sensibility and can also indicate more or less species (Jörger et al. 2012, Mirales & Vences, 2013), depending also on the sampling/population coverage. Unfortunately, opisthobranch sampling is usually suboptimal, i.e. not allowing the inclusion of a high number of specimens of putative species, and model assumptions may not be suited. Special properties and limitations of the different species delimitation analyses are being explored (e.g. Cartens et al. 2006, Dellicour & Flot, 2015), indicating that some recent most trustfull lines for the decision on the number of species are not necessarily the most accurate. Herein, potentially differing results on the number of species based on molecular data thus are compared, all together, to color/morphology as also ecological/biological evidences (Figure 4). A final step, integrating molecular and other evidences, should take in consideration the existence of syntopy, sympatry or allopatry of the different lineages delimited (Figure 4). Sympatry, and mostly syntopy, of lineages delimited under this integrative workflow strengthens the hypothesis of different species due to a presumptive evidence for their reproductive isolation (Jörger et al. 2014, Kekkonen & Hebert, 2014).
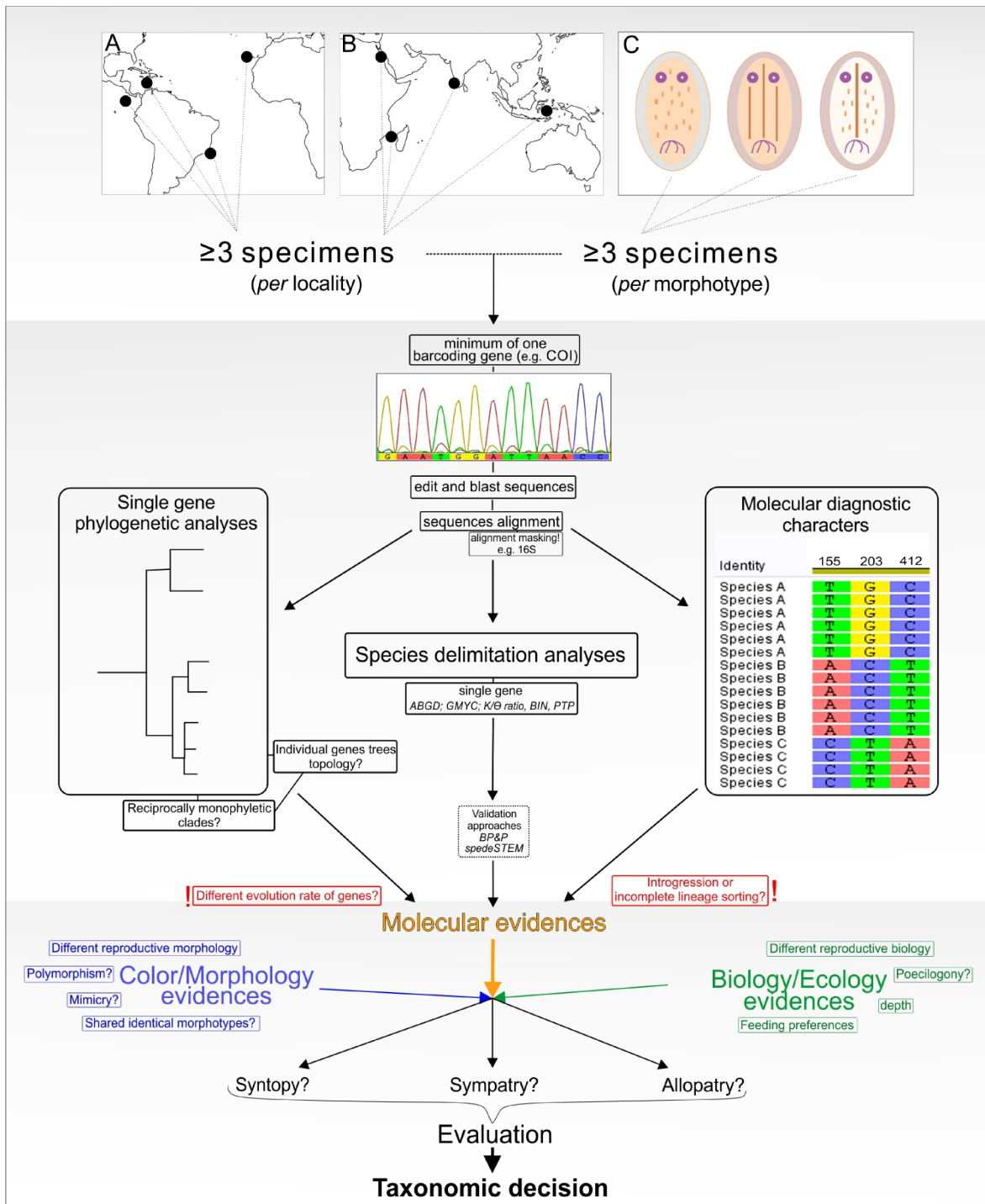
Figure 4. Proposed integrative taxonomic approach for conspicuous opisthobranchs and other non-microscopic marine, shallow water, invertebrates. Preliminary case selection may consider, for example, species with wide geographic distribution (A, B) or with color polymorphism (C). The minimum approach requires sequencing at least one barcoding gene of three specimens per morphotype/color morph. Proper sequences alignment is the basis for the phylogenetic (e.g. Maximum Likelihood) and species delimitation analyses (e.g. ABGD, GMYC), and for the detection of diagnostic nucleotide characters. Molecular evidences should then be considered together with color/morphological, and biological/ecological evidences and all together evaluated by the occurrence of syntopy, sympatry and allopatry of the different delimited groups.

This workflow does not invalidate the use of morphology in taxonomy but proposes the use, when possible, of molecular data as a first tool to a preliminary delimitation of species. The proposed minimum requirements try to balance the quantity and quality of

data needed for meaningful application of analyses on one hand with the limited availability of suitable material, the rarity of some species, and the economy in time, energy and costs during lab procedures and analyses. The latter aspect is particularly relevant for not well developed countries, which host most of the world marine biodiversity, but have limited scientific structure and budget. The proposed workflow is a general approach and directed mainly to cases where traditional taxonomy has potentially failed. However, every case has its own taxonomic history. Along the time, taxonomists may have different opinions and hypotheses on the number of species in a group of similar species, as is the case of the *Felimida clenchi* complex (Padula et al. in prep.). These different hypotheses are supported or rejected by a range of evidences. In fact, competing rather than fully supporting or at least compatible lines of evidences are common in molluscan taxonomy. Species delimitation by accumulation or congruence of evidence as proposed by Padial et al. (2010) thus cannot easily applied to problematic groups or complex taxonomic questions. Furthermore, not solely the number of evidences for a certain hypothesis relative to others ("democratic approach") is relevant, but also their individual power. Conflicting evidences for different hypotheses on the number of species thus need to be shown and evaluated case by case, and a decision needs to be reached. This process of comparing and evaluating partly conflictive evidences can be visualized as shown in Figure 5, making final taxonomic decisions transparent. In summary, newly integrative approaches, as following the workflow proposed above, result in the inference of new evidences. These new evidences, in general, allow a more reliable taxonomic decision (Padula et al. in prep.). As every case has its own taxonomic history and a more or less complex picture of competing evidences, these should be made transparent, facilitating revision in the light of future data.
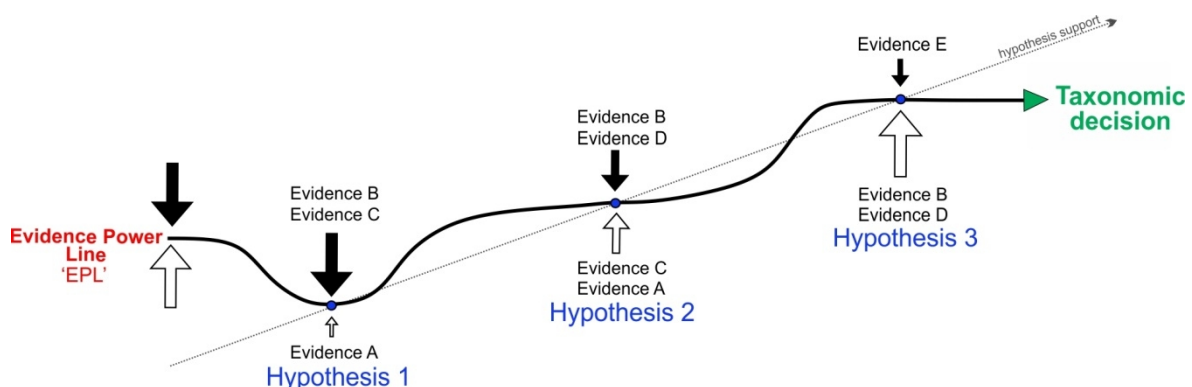
Figure 5. The 'Evidence Power Line' (EPL), a graphical resume of the path to the taxonomic decision. Different hypotheses (e.g. in the number of species) are represented in the line (blue spots) with supporting (white arrows) and rejecting (black arrows) evidences. A same evidence may have different weights and also support or reject different hypotheses. The higher is the hypothesis arranged at the line, the higher is its support. Taxonomists should evaluate the evidences obtained from the different analyses to support their taxonomic decision.

The evidence power line (EPL) (Figure 5) is particularly usefull for historically problematic taxonomic cases, which accumulate evidences on many hypotheses on the number of species along the time. The EPL visualizes the way towards the taxonomic decision in complicated taxonomic histories in the light of the new evidences obtained from integrative taxonomic approaches.

4.4 Polychromatism and mimetism

One of the most relevant results in the different cases studied along this thesis deals about body color pattern. While different, not necessarily closely related opisthobranch species can show very similar or even identical color morphs, e.g. in the *Navanax aenigmaticus* complex (Ornelas-Gatdula et al. 2012), *Cratena* spp. (Padula et al., 2014a) and *Pleurobranchus* spp. (Goodheart et al. 2015), a single species can be extremely variable in color pattern, as is the case of *Pleurobranchus areolatus* (see chapter 9, fig. 18) and, in particular, *Felimida binza* (see chapter 11, figs. 8 and 9). For the first time, the existence of extreme color polymorphism in brightly colored sea slugs species is supported by molecular data and integrative taxonomic approaches. This extreme color polymorphism has direct implication to opisthobranch taxonomy, because body color pattern is currently one of the main characters for the identification and diagnosis of the species (Gosliner et al. 2008). Similar body color pattern occur among groups of sympatric species (chapter 11) and are evident cases of Müllerian mimicry, as discussed by Ros (1976) and Edmunds (1981, 1987). However, the origin of mimicry and mimetic color groups in sea slugs remains unexplored. Similar cases of polychromatism and mimicry in other chromatic groups of animals, such as butterflies, damselflies, cichlid fish and birds involves hybrid speciation and adaptive introgression (Sánchez-Guillén et al. 2005, Wellenreuther et al. 2014). The *Felimida clenchi* complex (see chapter 11) and the various color groups of sea slug species in the Indo-Pacific region (see Rudman 1991) represent interesting models for further, deeper studies in mimicry and origin of the wide color diversity in opisthobranchs and in the marine environment in general.

5. CONCLUSIONS AND OUTLOOK

'Testing traditional concepts' in biodiversity and taxonomy of Brazilian opisthobranchs, the present study revealed many new aspects, including new geographic records and undescribed species; the observation of novel anatomical details from a 3D microanatomical reconstruction; and evidences of cryptic diversity in almost all non-microscopic opisthobranch species studied. The main signal is clear: traditional taxonomy and previous biodiversity approaches on Brazilian opisthobranch species suffered from limited sampling of material and data available. Modern (micro) morphological techniques and molecular analyses provide a set of additional and powerful approaches for integrative taxonomy.

This thesis combined new field collections with applying traditional and modern taxonomic approaches on opisthobranchs. Specimens were discovered and observed, habitats and e.g. coloration of living animals were documented, preserved specimens dissected and classical features such as radulae and reproductive organs examined, and data compared with literature descriptions and museum material available. Existing species descriptions were thus supplemented, new species described according to current morphology-based practices, and biogeographic hypotheses reconsidered according to the new data available. This work thus is rooted in the taxonomic and nomenclatural history on Brazilian opisthobranchs and this is considered important for not losing track, but it obviously goes further. Based on the newly collected material, which was suitably fixed for molecular studies, and in collaboration with other researchers contributing material from other regions, a wide range of molecular phylogenetic and species delimitation analyses was used to test traditional species hypotheses on selected, potentially problematic taxa. In all cases studied under this integrative approach in this thesis, the novel hypotheses on the number of species differed from those from the traditional taxonomy. Usually, cryptic species were discovered by molecular analyses which, in some cases, could then be related to slight but consistent morphological differences, diagnosing these pseudo-cryptic species. I conclude that traditional and currently still applied practices in opisthobranch taxonomy are not generally adequate to resolve species limits, at least when referring to apparently widespread or variable "species". The species diversity of opisthobranchs thus is probably severely underestimated, in Brazil and on a global scale. Opisthobranch researcher may reconsider current morphology-based practices. Molecular characters results in a better initial delimitation of species than morphology, and consequently I propose a workflow that starts with molecular barcoding, but ends with considering any other potentially useful

characters, such as morphology, and other information, such as geographical or developmental.

While considering more data is usually beneficial, I also noted that different molecular species delimitation analyses may support quite different hypotheses, so there was no obvious way of integrating various, partly incompatible evidences. The herein proposed "evidence power line" is a trial to illustrate complex taxonomic histories and decisions. In my opinion, applying character based approaches, such as identifying diagnostic nucleotides, amino acids or indels, are a promising avenue to delimitate and describe even recently separated lineages, which otherwise would need exhaustive population genetic approaches (which are usually not feasible on marine invertebrates due to lacking material and unknown geographic ranges). A surprising, if not to say disturbing, discovery herein is the existence of highly similar and complex color patterns in not closely related sea slug species. I suggest that mimicry contributes to blur species limits, a phenomenon that could have major impact on the taxonomy of highly species-diverse Indo-Pacific sea slugs, which usually form color-groups.

6. ACKNOWLEDGEMENTS

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Wägele H, Klussmann-Kolb A (2005) Opisthobranchia (Mollusca, Gastropoda) - more than just slimy slugs. Shell reduction and its implications on defence and foraging. *Frontiers in Zoology* **2**: 1-18.

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Wägele H, Klussmann-Kolb A, Verbeek E, Schrödl M (2014) Flashback and foreshadowing—a review of the taxon Opisthobranchia. *Organisms Diversity & Evolution*. doi 10.1007/s13127- 013-0151-5.

Wellenreuther M, Svensson E, Hansson B (2014) Sexual selection and genetic colour polymorphisms in animals. Molecular Ecology **23**: 5398-5414.

Wilson NG, Lee MSY (2005) Molecular phylogeny of *Chromodoris* (Mollusca, Nudibranchia) and the identifcation of a planar spawning clade. *Molecular Phylogenetics & Evolution* **363**:722-727.

Wilson NG, Schrödl M, Halanych KM (2009) Ocean barriers and glaciation: evidence for explosive radiation of mitochondrial lineages in the Antarctic sea slug *Doris kerguelenensis*. *Molecular Ecology* **18**: 965-984.

Yonow (2015) Sea Slugs: Unexpected Biodiversity and Distribution. Pp. 531-550 in *The Red Sea. The Formation, Morphology, Oceanography and Environment of a Young Ocean Basin*. Rasul NMA, Stewart ICF (eds.) Springer.

Zapata F, Wilson NG, Howison M, Andrade SCS, Jörger KM, Schrödl M, Goetz FE, Giribet G. Dunn, CW (2014) Phylogenomic analyses of deep gastropod relationships reject Orthogastropoda. *Proceedings of the Royal Society B: Biological Sciences* **281**: 1471-2954.

8. APPENDIX

8.1 DECLARATION OF OWN CONTRIBUTION AS CO-AUTHOR

Chapter 1

Padula V, Bahia J, Vargas C, Lindner A (2011) Mollusca, Nudibranchia: new records and southward range extensions in Santa Catarina, Southern Brazil. *Check List* **7**: 806-808.

I planned the work; collected, photographed and identified specimens; drafted and submitted the manuscript; revised the manuscript after reviewers and editor opinion.

Chapter 2

Padula V, Bahia J, Correia MD, Sovierzoski HH (2012) New records of opisthobranchs (Mollusca: Gastropoda) from Alagoas, Northeastern Brazil. *Marine Biodiversity Records* **5**: e57.

I planned the work; collected, photographed and identified specimens; drafted and submitted the manuscript; revised the manuscript after reviewers and editor opinion.

Chapter 3

Camacho-García Y, Pola M, Carmona L, **Padula V**, Villani G, Cervera L (2014) Diversity and distribution of the heterobranch sea slug fauna on the Caribbean of Costa Rica. *Cahiers de Biologie Marine* **55**: 109-127.

I collected, photographed and identified specimens; wrote partially the manuscript; revised the manuscript after reviewers and the journal editor opinion.

Chapter 4

Padula V, Wirtz P, Schrödl M (2014) Heterobranch sea slugs (Mollusca: Gastropoda) from Ascension Island, South Atlantic Ocean. ***Journal of the Marine Biological Association of the United Kingdom***. DOI: http://dx.doi.org/10.1017/S0025315414000575

I identified the specimens; conducted morphological study and performed SEM micrographs; drafted, submitted and revised the manuscript.

Chapter 5

Brenzinger B, **Padula V**, Schrödl M (2013) Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug

Pluscula cuica Marcus, 1953 from Brazil (Gastropoda: Euopisthobranchia: Philinoglossidae). *Organisms, Diversity & Evolution* **13**: 33-54.

I performed histological sections and photographs and part of the 3D reconstruction; I discussed and improved the manuscript with the co-authors.

Chapter 6

Ornelas-Gatdula E, Camacho-García Y, Schrödl M, **Padula V**, Hooker Y, Gosliner TM, Valdés Á (2012) Molecular systematics of the ʻ*Navanax aenigmaticus*ʼ species complex (Mollusca, Cephalaspidea): coming full circle. *Zoologica Scripta* **41**: 374-385.

I performed DNA extraction, PCR preparation and edition of sequences of ZSM samples; I discussed and improved the manuscript.

Chapter 7

Padula V, Araújo AK, Matthews-Cascon H, Schrödl M (2014) Is the Mediterranean nudibranch *Cratena peregrina* (Gmelin, 1791) present in the Brazilian coast? Integrative species delimitation and description of *Cratena minor* n. sp. *Journal of Molluscan Studies* **80**: 575-584.

I planned the work; performed DNA extraction, PCR preparation, edition of sequences, phylogenetic and species delimitation analysis; drafted, wrote and revised the manuscript.

Chapter 8

Pola M, **Padula V**, Gosliner TM, Cervera JL (2014) Going further on an intricate and challenging group of nudibranchs - description of five new species and a more complete molecular phylogeny of the subfamily Nembrothinae (Polyceridae). *Cladistics* **30**: 607-634.

I collected, photographed and dissected Brazilian specimens; discussed the data with the co-authors; drafted part of the results; suggested the title of the manuscript; revised the manuscript.

Chapter 9

Goodheart JA, Camacho-García Y, **Padula V**, Schrödl M, Cervera JL, Gosliner TM, Valdés Á (2015) Systematics and biogeography of *Pleurobranchus* Cuvier, 1804 sea slugs (Mollusca: Heterobranchia: Nudipleura: Pleurobranchidae). *Zoological Journal of the Linnean Society*. DOI: 10.1111/zoj.12237.

I planned the work with co-authors, performed DNA extraction, PCR preparation and the edition of sequences of some samples; discussed and improved the manuscript; revised and improved the manuscript after the reviewer's opinion.

Chapter 10

Ortigosa D, Pola M, Carmona L, **Padula V**, Schrödl M, Cervera JL (2014) Redescription of *Felimida elegantula* (Philippi, 1844) and a preliminary phylogeny of the European species of *Felimida* (Chromodorididae). *Journal of Molluscan Studies* **80**: 541-550.

I planned the work with co-authors, performed DNA extraction, PCR preparation and the edition of sequences of some samples; discussed and improved the manuscript; revised and improved the manuscript after the reviewer's opinion.

Chapter 11

Padula V, Bahia J, Stöger I, Camacho-García Y, Malaquias M, Cervera JL & Schrödl M. (in prep.). A test for color based taxonomy in nudibranchs: molecular phylogeny and species delimitation of the *Felimida clenchi* (Mollusca: Chromodorididae) species complex. *Molecular Phylogenetics and Evolution*.

I planned the work; collected and photographed many specimens; performed DNA extraction, PCR preparation, edition of sequences, phylogenetic and species delimitation analyses; drafted the manuscript.

Munich, May 2015

I hereby confirm the above statement,

Vinicius Padula Anderson _____

PD Dr. Schrödl Michael _____

8.2 CURRICULUM VITAE

Personal data

Name	Vinicius Padula Anderson
E-mail	viniciuspadula@yahoo.com
Birth	July 06, 1982 in Rio de Janeiro, Brazil

Studies

<u>PhD</u>

04.2011-present   *Morpho-species versus genetic diversity - a case study on South American opisthobranchs gastropods*. Ludwig-Maximilians-Universität München (LMU) Supervisor: PD Dr. Michael Schrödl, LMU and Zoologische Staatssammlung München (ZSM). Project funded by the Brazilian National Council for Scientific and Technological Development (CNPq) and the Deutscher Akademischer Austauschdienst (DAAD).

<u>Master</u>

07.2008-07.2009   Biological Sciences (Zoology)
Taxonomic revision of the genus Hypselodoris *Stimpson, 1855 (Mollusca, Gastropoda, Nudibranchia) from Brazil*. Museu Nacional / Universidade Federal do Rio de Janeiro, Brazil. Supervisor: Prof. Dr. Alexandre Pimenta. Funded by Brazilian National Council for Scientific and Technological Development (CNPq).

<u>Bachelor/Graduation</u>

07.2002-07.2007   Biological Sciences (Marine Biology)
Taxonomic characterization of species of the suborder Aeolidina (Mollusca, Gastropoda, Nudibranchia) from Praia das Conchas, Cabo Frio, Rio de Janeiro, Brazil. Universidade Federal do Rio de Janeiro, Brazil. Supervisor: Prof. Dr. Alexandre Pimenta.

Complementary courses received

2006	Zoological Illustration. XXVI Brazilian Congress of Zoology, Londrina, Brazil
2004	Metazoan Phylogeny. XXV Brazilian Congress of Zoology, Brasília, Brazil

Technical courses received

Scuba diving. PDIC: OpenWater, ID: 102410, Brazil, 2005.

Scuba diving. CMAS / CBPDS: P1-11906.

Research lab work experience

Present-2011	Zoologische Staatssammlung München. Malacology Section.
2010-2006	Museu Nacional / Universidade Federal do Rio de Janeiro, Brazil. Malacology Section.
2006	Natural History Museum of Los Angeles County, USA. Malacology Section.
2006-2003	Universidade Federal do Rio de Janeiro, Brazil. Malacology Section.
2005-2004	Universidade Federal do Rio de Janeiro, Brazil. Fishery Biology and Technology Section.

Tutorship and teaching experience

2013-2012	Discipline Malacology / LMU. - Molecular lab techniques.
2008	Course *Diversity of nudibranchs and marine flatworms.* XII BioWeek from Universidade Federal do Rio de Janeiro, Brazil.
2006	Course *The life on rocky shores*. X BioWeek from Universidade Federal do Rio de Janeiro, Brazil.
2005	Discipline Zoology II, undergraduate course of Biological Sciences. Universidade Federal do Rio de Janeiro, Brazil. One semester.
2005	Program of monitored visits at the Centro de Biologia Marinha of Universidade de São Paulo (CEBIMar). One month.
2004	Discipline Vegetal Physiology, undergraduate course of Biological Sciences of the Universidade Federal do Rio de Janeiro, Brazil. One semester.

Student supervision

2013-present	Co-supervisor of the candidate Marlon Delgado Melo in his work for a Master degree on Systematics and Evolution, entitled "Levantamento taxonomico de moluscos opistobranquios (Mollusca: Gastropoda) no litoral do Rio Grande do Norte, Brasil" Universidade Federal do Rio Grande do Norte, Brazil.
2009	Co-supervisor of student Thalita Dionísio Belmonte in her work for Graduation degree on Biological Sciences, entitled "Spongivory by nudibranchs (Mollusca, Gastropoda) at the coast of Rio de Janeiro State", Universidade Santa Úrsula, Rio de Janeiro, Brazil, 2009.

Grants received

Present-2010	PhD studies. Deutscher Akademischer Austauschdienst (DAAD) and Brazilian National Council for Scientific and Technological Development (CNPq)
2014	Malacological Society of London, Travel Award
2013	Malacological Society of London, Travel Award
2009-2007	Master studies. National Counsel of Technological and Scientific Development (CNPq) Brazil
2006	Adams Foundation Internship in Biological Systematics, Los Angeles, USA.
2005	Tutorship Grant. Undergraduate discipline, Universidade Federal do Rio de Janeiro, Brazil

Awards received

2013	Unitas Malacologica. Award 'Student presentation of excellence'. World Congress of Malacology, Azores
2007	Brazilian Society of Malacology. Award 'Professor Maury Pinto de Oliveira' for scientific research excellence on Malacology
2006	Brazilian Society of Zoology. Award for the best academic work of the XXVI Brazilian Congress of Zoology
2006	Brazilian Society of Zoology. Third place in the Zoological Photography contest of the XXVI Brazilian Congress of Zoology

Reviews for journals

Bulletin of Marine Science

Helgoland Marine Research

Journal of Conchology

Journal of Ecology and Natural Environment

Journal of Molluscan Studies

Journal of the Marine Biological Association of the United Kingdom

Marine Biodiversity Records

Spixiana

The Nautilus

Zootaxa

Munich, May 2015

Vinicius Padula Anderson _____

LIST OF PUBLICATIONS

Peer-reviewed contributions

1. Goodheart JA, Camacho-García Y, **Padula V**, Schrödl M, Gosliner TM, Valdés Á (2015) Systematics and biogeography of *Pleurobranchus* Cuvier, 1804 sea slugs (Mollusca: Heterobranchia: Nudipleura: Pleurobranchidae). *Zoological Journal of the Linnean Society*. DOI: 10.1111/zoj.12237

2. **Padula V**, Araújo AK, Matthews-Cascon H, Schrödl M (2014) Is the Mediterranean nudibranch *Cratena peregrina* (Gmelin, 1791) present in the Brazilian coast? Integrative species delimitation and description of *Cratena minor* n. sp. *Journal of Molluscan Studies* **80**: 575-584.

3. Pola M, **Padula V**, Gosliner TM, Cervera JL (2014) Going further on an intricate and challenging group of nudibranchs - description of five new species and a more complete molecular phylogeny of the subfamily Nembrothinae (Polyceridae). *Cladistics* **30**: 607-634.

4. **Padula V** (2014) Nudibrânquios: taxonomia e diversidade na costa brasileira. *Boletim Informativo da Sociedade Brasileira de Zoologia* **109**: 5-7.

5. Bahia J, **Padula V**, Lavrado H, Quiroga S (2014) Taxonomy of Cotylea (Platyhelminthes: Polycladida) from Cabo Frio, southeastern Brazil, with the description of a new species**.** *Zootaxa* **3873**: 495-525.

6. Ortigosa D, Pola M, Carmona L, **Padula V**, Schrödl M, Cervera JL (2014) Redescription of *Felimida elegantula* (Philippi, 1844) and a preliminary phylogeny of the European species of *Felimida* (Chromodorididae). *Journal of Molluscan Studies* **80**: 541-550.

7. **Padula V**, Wirtz P, Schrödl M (2014) Heterobranch sea slugs (Mollusca: Gastropoda) from Ascension Island, South Atlantic Ocean. *Journal of the Marine Biological Association of the United Kingdom*.
DOI: http://dx.doi.org/10.1017/S0025315414000575.

8. Pereira FR, Santos MFC, Williams DE, Andersen RJ, **Padula V**, Ferreira AG, Berlinck RGS (2014) Rodriguesic acids, modified diketopiperazines from the gastropod mollusc *Pleurobranchus areolatus*. *Journal of the Brazilian Chemical Society* **25**: 788-794.

9. Camacho-García Y, Pola M, Carmona L, **Padula V**, Villani G, Cervera L (2014) Diversity and distribution of the heterobranch sea slug fauna on the Caribbean of Costa Rica. *Cahiers de Biologie Marine* **55**: 109-127.

10. Brenzinger B, **Padula V**, Schrödl M (2013) Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Gastropoda: Euopisthobranchia: Philinoglossidae). *Organisms, Diversity & Evolution* **13**: 33-54.

11. Sales L, Queiroz V, **Padula V**, Neves EG, Johnsson R (2013) New records of nudibranchs (Mollusca: Gastropoda) from Bahia State, northeastern Brazil. *Check List* **9**: 689-691.

12. **Padula V**, Valdés Á (2012) Phylogeny and biogeography of *Paradoris* (Nudibranchia, Discodorididae), with the description of a new species from the Caribbean Sea. *The Veliger* **51**: 165-176.

13. Pereira FR, Berlinck RGS, Rodrigues Filho E, Veloso K, Ferreira AG, **Padula V** (2012) Metabólitos secundários dos nudibrânquios *Tambja stegosauriformis*, *Hypselodoris lajensis* e *Okenia zoobotryon* e dos briozoários *Zoobotryon verticillatum* e *Bugula dentata* da Costa do Brasil. *Química Nova* **35**: 2194-2201.

14. **Padula V**, Bahia J, Correia MD, Sovierzoski HH (2012) New records of opisthobranchs (Mollusca: Gastropoda) from Alagoas, Northeastern Brazil. *Marine Biodiversity Records* **5**: e57.

15. Ornelas-Gatdula E, Camacho-García Y, Schrödl M, **Padula V**, Hooker Y, Gosliner TM, Valdés Á (2012) Molecular systematics of the `*Navanax aenigmaticus*´ species

complex (Mollusca, Cephalaspidea): coming full circle. *Zoologica Scripta* **41**: 374-385.

16. Bahia J, **Padula V**, Delgado M (2012) Five new records and morphological data of five polyclad species (Platyhelminthes: Turbellaria) from Rio Grande do Norte, northeastern Brazil. *Zootaxa* **3170**: 31-44.

17. **Padula V**, Bahia J, Vargas C, Lindner A (2011) Mollusca, Nudibranchia: new records and southward range extensions in Santa Catarina, Southern Brazil. *Check List* **7**: 806-808.

18. Sales L, Delgado M, Queiroz V, **Padula V**, Sampaio C, Johnsson R (2011) First record of *Flabellina dana* Millen and Hamann, 2006 (Mollusca: Nudibranchia) in the South Atlantic Ocean. *Check List* **7**: 880-882.

19. Alvim J, **Padula V**, Pimenta AD (2011) First record of the genus *Onchidoris* (Gastropoda: Nudibranchia: Onchidorididae) from the South Atlantic Ocean, with the description of a new species from Brazil. *Journal of the Marine Biological Association of the United Kingdom* **91**: 505-511.

20. **Padula V**, Delgado M (2010) A new species of *Cerberilla* (Gastropoda: Nudibranchia: Aeolidiidae) from northeastern Brazil. *The Nautilus* **124**: 175-180.

21. DaCosta S, **Padula V**, Schrödl M (2010) A new species of *Hypselodoris* and a redescription of *Hypselodoris picta lajensis* (Nudibranchia: Chromodorididae) from Brazil. *The Veliger* **51**: 15-25.

22. Carbone M, Irace C, Costagliola F, Castelluccio F, Villani G, Calado G, **Padula V**, Cimino G, Cervera JL, Santamari R (2010) A new cytotoxic tambjamine alkaloid from the Azorean nudibranch *Tambja ceutae*. *Bioorganic & Medicinal Chemistry Letters* **20**: 2668-2670.

23. Bahia J, **Padula V** (2009) First record of *Pseudoceros bicolor* and *Pericelis cata* (Platyhelminthes: Polycladida) from Brazil. *Marine Biodiversity Records* **2**: e86.

24. Malaquias M, Calado G, **Padula V**, Villani G, Cervera L (2009) Molluscan diversity in the North Atlantic Ocean: new records of opisthobranch gastropods from the Archipelago of the Azores. *Marine Biodiversity Records* **2**: e38.

25. **Padula V** (2008) Notes on the morphology of *Elysia subornata* and *Oxynoe antillarum* (Mollusca, Opisthobranchia, Sacoglossa) from the state of Rio de Janeiro, Brazil. *Strombus* **15**: 19-25.

26. **Padula V**, Santos FN (2006) Three new records of Nudibranchia (Mollusca, Gastropoda) - additions on the Brazilian biodiversity. *Biociências* **14**: 214-220.

27. **Padula V**, Absalão RS (2005) Primeiro registro de *Babakina festiva* (Roller, 1972) (Mollusca: Nudibranchia) no Atlântico Sul. *Biociências* **13**: 99-101.

Book chapters
28. Cunha CM, **Padula V**, Magalhães ARM, Ferreira FM, Ferreira JF (2014) Moluscos In: Alberto Lindner (org.) *Vida Marinha de Santa Catarina*. Editora da UFSC, Florianópolis. Pp 38-51.

29. Vianna M, Andrade-Tubino MF, Keunecke KA, Andrade AC, Silva-Junior DR, **Padula V** (2012) Estado atual do conhecimento sobre a ictiofauna In: Maria de Fátima Guadalupe Meniconi (org.). *Baía de Guanabara: Síntese do Conhecimento Ambiental*. Petrobrás, Rio de Janeiro. Pp. 169-195.

Scientific meetings contributions (between 2011-2014)
30. **Padula V**, Schrödl M (2014) Do you believe in colour? Cases of cryptic diversity in Atlantic sea slugs (Gastropoda: Heterobranchia). In: Abstracts of the *Mollusca Forum 2014*, London. (Oral presentation)

31. Deneb Ortigosa J, **Padula V**, Carmona L, Pola M, Schrödl M, Cervera JL (2014) Looking like a comb! Preliminary phylogeny of the genus *Felimida*. In: Abstracts of the *7th Congress of the European Malacological Societies*, Cambridge. (Poster)

32. Deneb Ortigosa, Pola M, **Padula V**, Carmona L, Calado G, Cervera JL (2014) Yellow spots and stripes over the blue, is the chromodorid nudibranch *Felimare picta* (Schultz in Philippi, 1836) a complex of species? In: Abstracts of the *Mollusca 2014 "El Encuentro de las Américas"*, Mexico City. (Oral presentation)

33. Correia MD, Bahia J, **Padula V**, Sovierzoski HH (2014) Novos registros e nova espécie de Polycladida (Platyhelminthes, Rhabditophora) para os ecossistemas recifais do estado de Alagoas. In: Abstracts of the ***Congresso Brasileiro de Oceanografia***, Itajaí. (Poster)

34. Enayati KK, Carmona L, **Padula V**, Schrödl M, Cervera JL (2013) *Aeolidia papillosa* (Linnaeus, 1761), single species or a cryptic species complex? A morphological and molecular study. In: Abstracts of the *World Congress of Malacology*, Ponta Delgada. *Açoreana* supplement **8**: 175-176. (Oral presentation)

35. Ortigosa D, **Padula V**, Pola M, Cervera JL (2013) Redescription of an uncommon Mediterranean Chromodorididae: *Felimida elegantula* (Philippi, 1844). In: Abstracts of the *World Congress of Malacology*, Ponta Delgada. *Açoreana* supplement **8**: 191-192. (Poster)

36. **Padula V**, Stöger I, Camacho-García Y, Brown J, Poursanidis D, Malaquias M, Cervera JL, Schrödl M (2013) A colorful question: the nudibranch ´*Felimida clenchi* species complex´ investigated through molecular markers. In: Abstracts of the *World Congress of Malacology*, Ponta Delgada. *Açoreana* supplement **8**: 178. (Oral presentation)

37. **Padula V**, Schrödl M (2013) The presence of the Mediterranean nudibranch *Cratena peregrina* in the Brazilian coast - evaluation using a mitochondrial and a nuclear molecular marker. In: Abstracts of the *World Congress of Malacology*, Ponta Delgada. *Açoreana* supplement **8**: 192. (Poster)

38. **Padula V**, Cunha CM, Bahia J, Tibiriçá Y, Lima PO, Saad L, Correia MD, Sovierzoski HH, Malaquias M, Tocino LS, Gómez JCG, Simone LRL, Cervera JL (2013) Following sea slugs: results of a recent opisthobranch expedition in the Northeastern

and Southeastern Brazilian coasts. In: Abstracts of the **World Congress of Malacology**, Ponta Delgada. *Açoreana* supplement **8**: 197. (Poster)

39. Pola M, **Padula V**, Cervera JL (2013) Going further on Nembrothinae – description of four new species and redescription of *Tambja divae*. 2013. In: Abstracts of the **World Congress of Malacology**, Ponta Delgada. *Açoreana* supplement **8**: 190-191. (Poster)

40. **Padula V**, Camacho-García Y, Malaquias M, Stöger I, Cervera JL, Schrödl M (2012) A colorful question: the chromodorid ´*Felimida clenchi*´ species complex investigated through COI. In: Abstracts of the *4th International Workshop on Opisthobranchs*, Santa Cruz (USA). (Oral presentation)

41. Camacho-García Y, Pola M, Carmona L, **Padula V**, Villani G, Cervera L (2012) Updating the Caribbean fauna of "opisthobranchs" (Mollusca, Gastropoda) of Costa Rica. In: Abstracts of the **Congresso Português de Malacologia**, Bragança. (Poster)

42. Delgado M, **Padula V**, D'Oliveira RG, Santos FN, Bahia J (2011) Diversidade e novos registros de opistobrânquios (Mollusca: Gastropoda) no litoral do Rio Grande do Norte, Brasil. In: Abstracts of the **XXII Encontro Brasileiro de Malacologia**, Fortaleza. (Oral presentation)

43. Oliveira LS, Melo MD, Araújo VQ, **Padula V**, Sampaio CLS, da Silva RJT (2011) *Flabellina dana* Millen & Hamann, 2006 (Mollusca, Nudibranchia): primeiro registro para o Oceano Atlântico Sul. In: Abstracts of the **XXII Encontro Brasileiro de Malacologia**, Fortaleza. (Oral presentation)

44. Oliveira LS, Araújo VQ, Padula V, Sampaio CLS, Neves EG, da Silva RJT (2011) Primeiro registro de *Navanax aenigmaticus* (Opisthobranchia, Aglajidae) para o estado da Bahia, Brasil. In: Abstracts of the **XXII Encontro Brasileiro de Malacologia**, Fortaleza. (Oral presentation)

Munich, May 2015

Vinicius Padula Anderson _____

Eidesstattliche Versicherung und Erklärung

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 06.05.2015

(Vinicius Padula Anderson)

Erklärung

Hiermit erkläre ich, dass die Dissertation **nicht** ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich **nicht** anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den 06.05.2015

(Vinicius Padula Anderson)