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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	Localitity	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

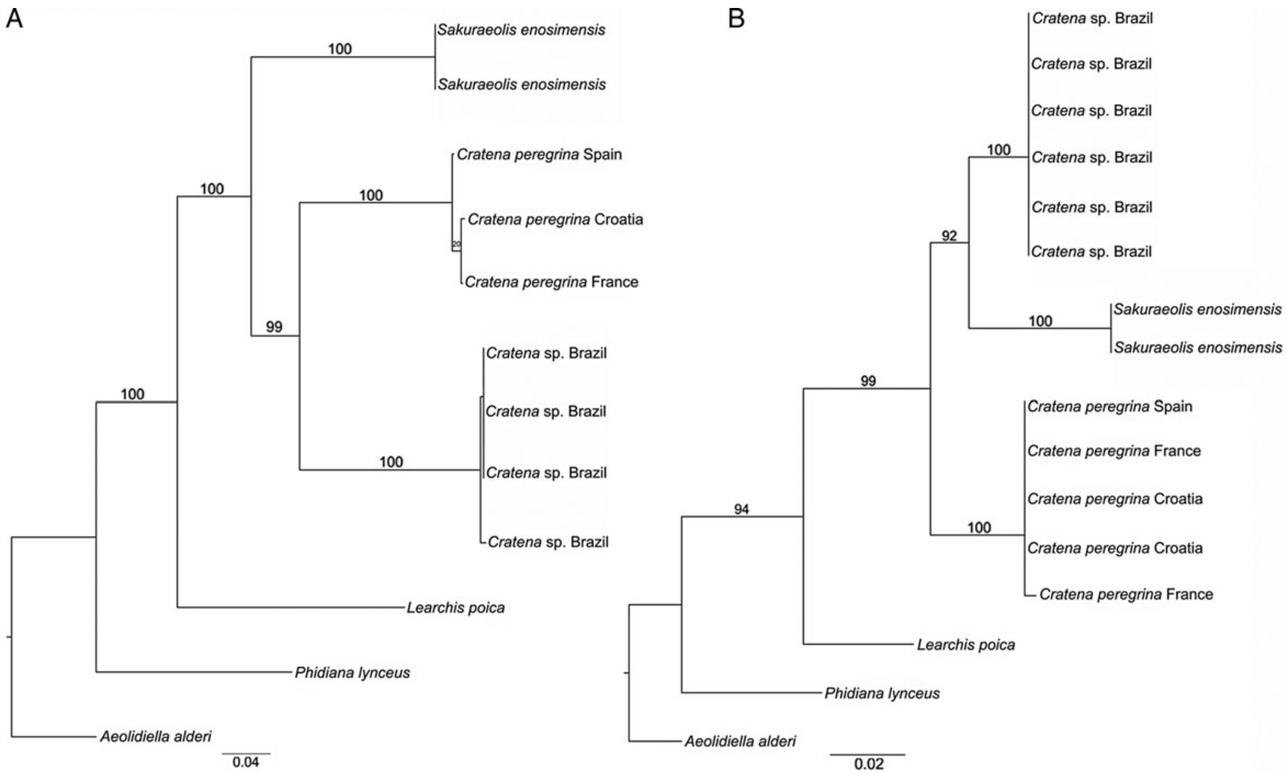

Figure 1. Maximum likelihood phylogenetic trees (1,000 replicates). Trees were rooted using *Aeolidiella alderi* as outgroup. **A.** Based on mitochondrial COI sequences. **B.** Based on nuclear H3 sequences. Bootstrap support values are shown above branches.

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.

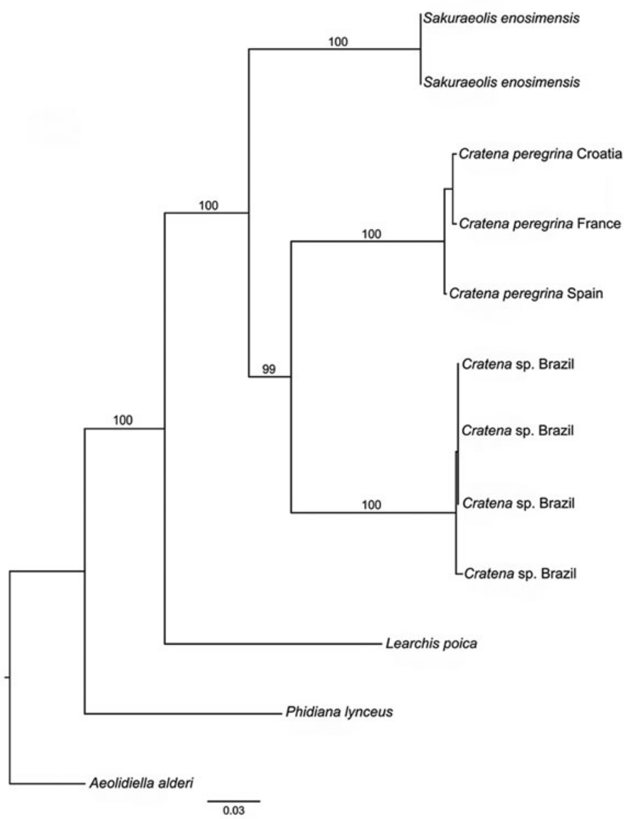

Figure 2. Maximum likelihood phylogenetic tree (1,000 replicates) rooted using *Aeolidiella alderi* as outgroup. Based on cytochrome *c* oxidase subunit I and H3 concatenated sequences. Bootstrap support values are shown above branches.

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*.,

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.

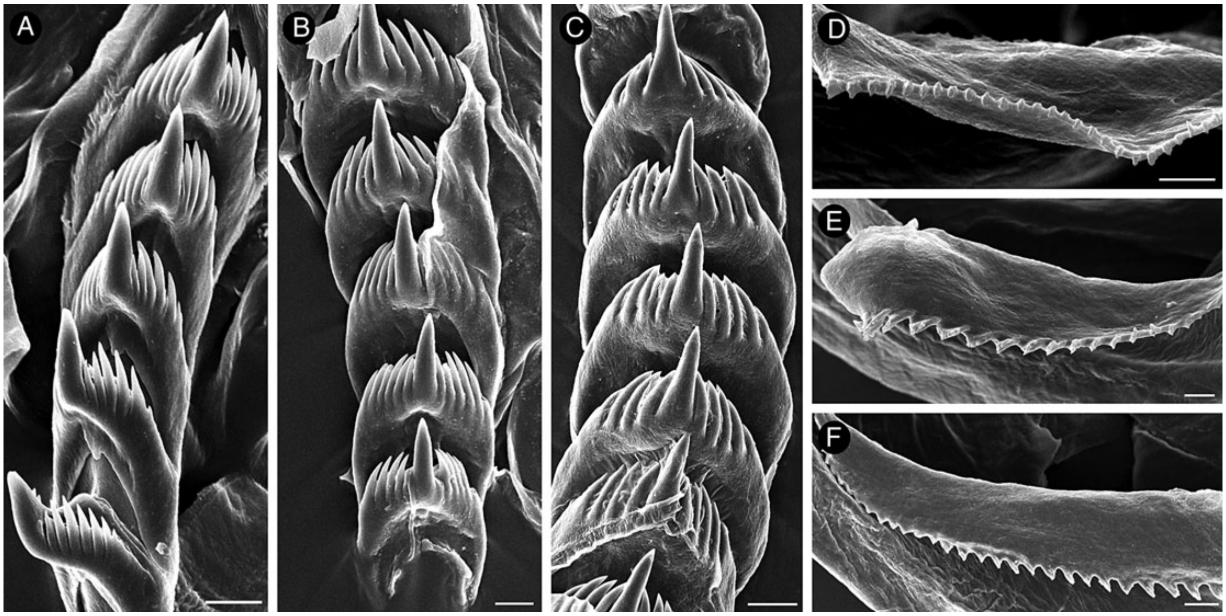

Figure 4. SEM micrographs. **A.** Rachidian teeth of *Cratena minor* n. sp. (ZSM Mol 20110345). **B, C.** Rachidian teeth of *C. peregrina* from Croatia (ZSM Mol 20100125) and France (ZSM Mol 20020957), respectively. **D.** Border of jaw of *C. minor* n. sp. (ZSM Mol 20110345). **E, F.** Border of jaw 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.

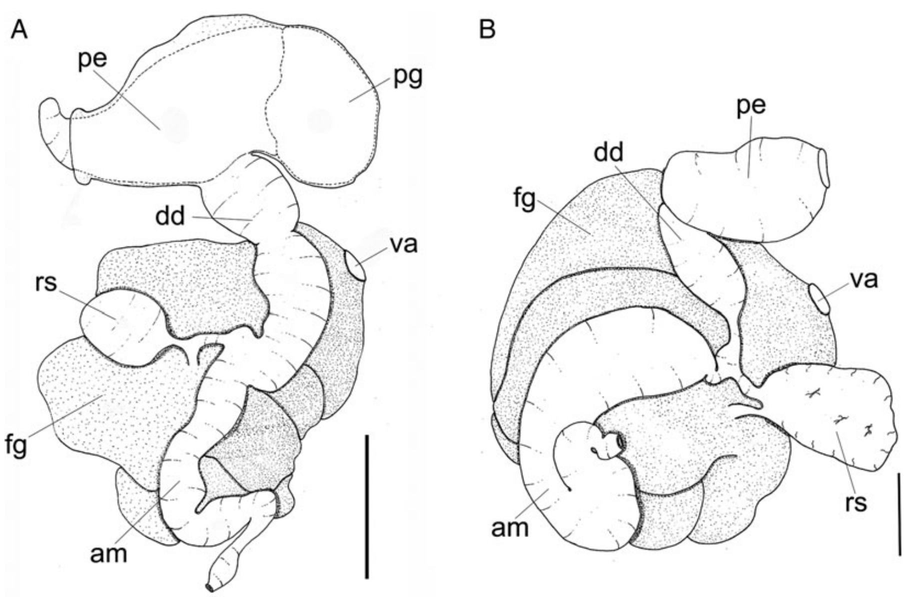

Figure 5. Reproductive system. **A.** *Cratena minor* n. sp. (CMPHRM 3728B). **B.** *C. peregrina* (ZSM Mol 20130772). Abbreviations: am, ampulla; dd, deferent duct; fg, female gland; pe, penis; pg, penial gland; rs, receptaculum seminis; va, vagina. Scale bars = 0.5 mm.

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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