



# Untangling the *Spurilla neapolitana* (Delle Chiaje, 1841) species complex: a review of the genus *Spurilla* Bergh, 1864 (Mollusca: Nudibranchia: Aeolidiidae)

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*Spurilla neapolitana* (Delle Chiaje, 1823) was considered to be a species with a broad geographic range and substantial colour variability; however, analyses of mitochondrial and nuclear gene data revealed that it is a complex of five distinct species. Further anatomical and morphological examinations determined that coloration is one of the main diagnostic traits for all five species, although some display substantial colour pattern variation. As a result of this study, *S. neapolitana* is determined to be restricted to the Mediterranean and eastern Atlantic. *Spurilla sargassicola* Bergh, 1871 from the Caribbean is redescribed and confirmed as a valid species. The name *Spurilla braziliana* MacFarland, 1909 is retained for western Atlantic and Pacific populations. Two new species are described herein. *Spurilla onubensis* sp. nov. occurs in Europe, with a range overlapping that of *S. neapolitana*. Finally, *Spurilla dupontae* sp. nov. is found in the Bahamas.

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## INTRODUCTION

*Spurilla neapolitana* (Delle Chiaje, 1841) is the type species of the genus *Spurilla*, and is probably one of the most common and widespread species within Aeolidiidae. Since its original description by Delle Chiaje (1841), this species has been studied widely

by numerous authors (Bergh, 1877; Trinchese, 1878; Bergh, 1882; Vayssière, 1888; Engel, 1925; Pruvot-Fol, 1953, 1954; Swennen, 1961; Ev. Marcus & Er. Marcus, 1967; Bebbington & Thompson, 1968; Gosliner, 1980; Schmekel & Portmann, 1982; García-Gómez & Cervera, 1985). It is currently accepted that *S. neapolitana* has a broad colour range and intraspecific variation in jaw denticulation and the shape of the central cusp. Furthermore, according

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to the literature, *S. neapolitana* is a 'globe-trotting nudibranch' (Willan, 2006), reported from the Mediterranean (where the type locality is located; Trinchese, 1878; Vayssi re, 1888; Luque, 1983, 1986; S nchez-Tocino, Oca na & Garc a, 2000), the eastern Atlantic (Garc a-G mez & Cervera, 1985; Calado *et al.*, 1999, 2003; Malaquias & Morenito, 2000), the western Atlantic (Padula, 2007; Dom nguez, Troncoso & Garc a, 2008; Garc a-Garc a, Dom nguez & Troncoso, 2008), the Caribbean Sea (Vald s *et al.*, 2006), and the Pacific Ocean (Gosliner, 1980; Hamatani, 2000; Camacho-Garc a, Gosliner & Vald s, 2005; Willan, 2006; Uribe & Pacheco, 2012).

Several synonyms have been attributed to this species: *Eolis sargassicola* Bergh, 1871; *Eolis alderiana* Deshayes & Fr dol, in Fr dol 1865; *Flabellina neapolitana* Costa, 1866; *Flabellina inornata* Costa, 1866; *Eolis conspersa* Fischer, 1869; *Spurilla braziliana* MacFarland, 1909; *Eolidina gabriellae* Vannucci, 1952; *Spurilla dakariensis* Pruvot-Fol 1953; *Spurilla mograbina* Pruvot-Fol, 1953, and *Spurilla vayssi rei* Garc a-G mez & Cervera, 1985 (Garc a-G mez & Cervera, 1985; Garc a-Garc a *et al.*, 2008; CLEMAM, 2012). Although most of these names are generally accepted as junior synonyms of *S. neapolitana*, the status of *S. sargassicola* is still in dispute. Recently, Vald s *et al.* (2006: p. 270) defended the validity of this species by comparing specimens of *S. neapolitana* and *S. sargassicola* in the same photograph. Moreover, the geographical range of *S. sargassicola* has been recently extended southwards by Sanvicente-A norve *et al.* (2012).

Based on data from three molecular markers, Carmona *et al.* (2013) clarified some important aspects of the systematics of *Spurilla*. That study confirmed that *S. sargassicola* is not a junior synonym of *S. neapolitana* but rather its sister species. Carmona *et al.* (2013) resurrected the name *S. braziliana* for western Atlantic populations previously considered to be *S. neapolitana*, thus rejecting the hypothetical amphiatlantic status of *S. neapolitana*.

In this paper, we attempt to further clarify the taxonomic status of different populations of *S. neapolitana* and closely related species based on molecular data from two mitochondrial (cytochrome *c* oxidase subunit I, *COI*; 16S rRNA, 16S) and one nuclear (histone 3, *H3*) gene, as well as anatomical features such as external morphology, radula, and reproductive system. Specimens covering the entire range of *S. neapolitana s.l.* have been included in this study. Another goal of this study is to review the pertinent literature in order to determine the valid names for members of the species complex. Finally, two new species of the genus *Spurilla* are described.

## MATERIAL AND METHODS

### LITERATURE REVIEW

A comprehensive review of the literature was conducted to determine the valid names for the species recognized in the molecular and morphological analyses. After the description of the type species, all available names for *Spurilla* species are organized and discussed in this paper according to the year of publication. In the synonymy lists, references to the original descriptions of the valid names and all synonyms (basionyms and primary synonyms), and the first proposed change of binomen (secondary synonyms), are included, but subsequent references are not.

### SOURCE OF SPECIMENS AND MORPHOLOGY

Samples were collected by scuba-diving with standard sampling techniques for opisthobranchs, or were obtained by loans from museum collections. Whenever possible, two or more specimens of each species were examined anatomically. Specimens were dissected by dorsal incision. Their internal features were examined and drawn under a stereoscopic microscope with the aid of a camera lucida. Special attention was paid to the morphology of the reproductive system and oral and salivary glands. The buccal mass was removed and dissolved in 10% sodium hydroxide until the radula was isolated from the surrounding tissue. The radula was then rinsed in water, dried, and mounted for examination under a scanning electron microscope (SEM).

Voucher specimens are held either at the California Academy of Sciences (CASIZ; San Francisco, CA, USA), Museo Nacional de Ciencias Naturales (MNCN; Madrid, Spain), Natural History Museum of Los Angeles County (LACM; Los Angeles, CA, USA), California State Polytechnic University Invertebrate Collection (CPIC; Pomona, CA, USA), Museu de Zoologia da Universidade S o Paulo (MZSP; S o Paulo, Brazil), University Museum of Bergen (ZMBN; Bergen, Norway), or Zoologische Staatssammlung M nchen (ZSM; Munich, Germany).

### MOLECULAR WORK

#### *Taxon sampling*

Two hundred and seventy eight specimens, including 47 species of Aeolidiidae, three species of Babakinidae, 32 species of Facelinidae, seven species of Flabellinidae, one species of Fionidae, three species of Piseinotecidae, and four species of Tergipedidae, were used for the phylogenetic analyses. A total of 218 specimens were successfully sequenced for *COI*, along with 243 for *16S*, and 256 for *H3*. Thirty-five

additional sequences were obtained from GenBank (see Table S1 for a full list of samples, localities, and voucher references).

*Tritonia challengeriana* Bergh, 1884 (= *Tritonia antarctica* Pfeffer in Martens & Pfeffer, 1886) was chosen as the out-group for its basal taxonomic position within Cladobranchia (Pola & Gosliner, 2010).

#### DNA extraction, amplification, and sequencing

DNA was extracted from foot tissue of specimens preserved in 70–100% ethanol, except in the case of small animals where the whole specimen was used. The DNeasy Blood & Tissue Kit (09/2001; Qiagen, Valencia, CA, USA) was used for DNA extraction.

Partial sequences of *COI*, *16S*, and *H3* were amplified by polymerase chain reaction (PCR) using the primers: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACAAAAATCA-3') (Folmer *et al.*, 1994) for *COI*; 16S ar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16S br-H (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.*, 1991) for *16S*; and H3AD5'3' (5'-ATGGCTCGTACCAAGCAGACVGC-3') and H3BD5'3' (5'-ATATCCTTRGGCATRATRGTTGAC-3') (Colgan *et al.*, 1998) for *H3*. These three gene regions are adequate for systematic studies of gastropods (e.g. Meyer, 2003; Williams & Reid, 2004; Dinapoli *et al.*, 2006; Frey & Vermeij, 2008; Malaquias & Reid, 2009; Pola & Gosliner, 2010; Pola, Camacho-Garcia & Gosliner, 2012; Carmona *et al.*, 2013; Claremont *et al.*, 2013; Eilertsen & Malaquias, 2013; Ohnheiser & Malaquias, 2013); however, several internal primers for *COI* and *H3* were designed for any specimens that did not amplify with the universal primers (see Carmona *et al.*, 2013 Table 1).

The PCRs were conducted in 50- $\mu$ L reactions containing 2  $\mu$ L of both forward and reverse primers (10  $\mu$ M), 5  $\mu$ L of deoxyribonucleotide (dNTP; 2 mM), a gene-dependent quantity of magnesium chloride (25 mM), 0.5  $\mu$ L of Qiagen DNA polymerase (5 U  $\mu$ L<sup>-1</sup>), 10  $\mu$ L of 'Q-solution' (5x), 5  $\mu$ L of Qiagen buffer (10x) (Qiagen Taq PCR Core Kit; cat. no. 201225), and 1  $\mu$ L of genomic DNA. The quantities of magnesium chloride used were 7  $\mu$ L for *COI* and *16S*, and 4  $\mu$ L for *H3*. The amplification of *COI* was performed with an initial denaturation for 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 30 s at 44 °C (annealing temperature), and 1 min at 72 °C, with a final extension of 7 min at 72 °C. The *16S* amplification began with an initial denaturation for 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 44 °C (annealing temperature), 1 min at 72 °C, with a final extension of 7 min at 72 °C. *H3* amplification was performed with an initial denaturation for 3 min at 95 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at 50 °C (annealing temperature), 2 min at 72 °C, with a final extension of 10 min at 72 °C.

Successful PCRs were purified by mixing 5  $\mu$ L of PCR product with 2  $\mu$ L of ExoSAP-IT (usb.affymetrix.com). Samples were incubated at 37 °C for 15 min, followed by an inactivation step at 80 °C for 15 min. Sequence reactions were run on a 3730XL DNA sequencer (Applied Biosystems). All new sequences have been deposited in GeneBank.

#### Sequence alignment and phylogenetic analyses

DNA sequences were assembled and edited using GENEIOUS PRO 4.7.6 (Drummond *et al.*, 2009). All sequences were checked for contamination with BLAST (Altschul *et al.*, 1990), implemented in the GenBank database. MAFFT (Katoh, Asiminos & Toh, 2009) was employed to align the sequences. The alignments were checked by eye using MacClade 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 the cut-off values from Carmona *et al.* (2013) as reference thresholds. Saturation was visually inspected in MEGA 5.0 (Tamura *et al.*, 2011) by plotting for all specimens, including the out-group, 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. No evidence of saturation was found even in third-codon positions.

The most variable regions from the *16S* rRNA alignment were removed using the default settings in GBLOCKS (Castresana, 2000). Excluding 'indel-rich' regions, the tree was in general poorly resolved, with lower node support. Therefore, final analyses were performed including all bases.

After primer removal, sequences of *COI*, *16S*, and *H3* were trimmed to 658, 463 (including gaps), and 327 base pairs, respectively.

Individual gene analyses and a concatenated analysis were performed. To test for conflicting phylogenetic signal between genes, the incongruence length difference test (ILD; Farris *et al.*, 1994) was conducted as the partition homogeneity test in PAUP\* 4.0b10 (Swofford, 2002). Test settings consisted of ten random stepwise additions (100 replicates) with tree bisection and reconnection (TBR) branch swapping.

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 GTR+I+G model was selected for the three genes.

Maximum-likelihood (ML) analyses were performed using the software RAxML 7.0.4 (Stamatakis, 2006), and node support was assessed with non-parametric

bootstrapping (BS) with 5000 replicates, random starting trees, and parameters estimated from each data set under the model selected for the original data set. Bayesian-inference (BI) analyses were conducted using MrBayes 3.1.2b (Ronquist & Huelsenbeck, 2003) for 50 million generations with two independent runs and a sampling frequency of 1000. The models implemented were those estimated with MrModeltest 2.3. The combined data set 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 1.4.1 (Drummond & Rambaut, 2007). For each analysis the first 12 500 trees were discarded ('burn-in' period) and node support was assessed with posterior probabilities (PPs). Only nodes supported by  $BS \geq 75$  and  $PP \geq 0.90$  are discussed. Identical haplotypes were collapsed using the program COLLAPSE 1.2 (Posada, 2004).

#### Genetic distances

In order to compare the genetic distances among specimens of Aeolidiidae, we calculated the pairwise uncorrected p distances for COI using PAUP\* 4.0b 10.0. The cut-off values from Carmona *et al.* (2013) were used as reference thresholds. All codon positions were considered for the analysis.

#### Population genetics analyses

The population structure for the COI haplotypes was studied performing a two-level hierarchical analysis of molecular variance (AMOVA) through ARLEQUIN 3.512 (Excoffier & Lischer, 2010). Analyses were conducted for populations for which more than one sequence was available. Samples were grouped by their geographical distribution and their position in the phylogenetic tree. *F*-statistic analogues, designated  $\Phi$ -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

### SYSTEMATICS

NUDIBRANCHIA CUVIER, 1817

CLADOBANCHIA WILLAN & MORTON, 1984

FAMILY AEOLIDIIDAE GRAY, 1827

#### Type species

*Eolis neapolitana* Delle Chiaje, 1841, by monotypy and subsequent designation by Bergh (1864).

Diagnosis of the genus *Spurilla* according to Bergh (1864):

*Rhinophori perfoliati*. *Podarium angulis fere rotundatis*. *Processus masticatorius madibulae parce et minutissime denticulatus*. *Radula dentibus pectinatis uniseriatis*.

Rhinophores perfoliate; foot corners rounded; masticatory process with minute denticles; radula pectinate and uniseriate.

*SPURILLA NEAPOLITANA* (DELLE CHIAJE, 1841)

(FIGS 1A–F, 2A–B, 3A)

*Eolis neapolitana* Delle Chiaje, 1841: vol. 6, pl. 73, figs 12–14; vol. 7, pl. 88, fig. 13–15 (1841); vol. 8, p. 7 (1844).

*Eolidia neapolitana* Vérany, 1846: 97.

*Facelina neapolitana* (Delle Chiaje, 1841): Bergh, 1861: 321.

*Spurilla neapolitana* (Delle Chiaje, 1841): Bergh, 1864: 205, plate IIB, figs 1–16.

*Eolis alderiana* Deshayes & Fré dol, in Fré dol 1865: pl. XVII, fig. 7.

*Flabellina neapolitana* Costa, 1866: 71, pl. 1, fig. 1.

*Flabellina inornata* Costa, 1866: 72, pl. 2, fig. 6.

*Spurilla inornata* (Costa, 1866): Carus, 1889–1893: 209.

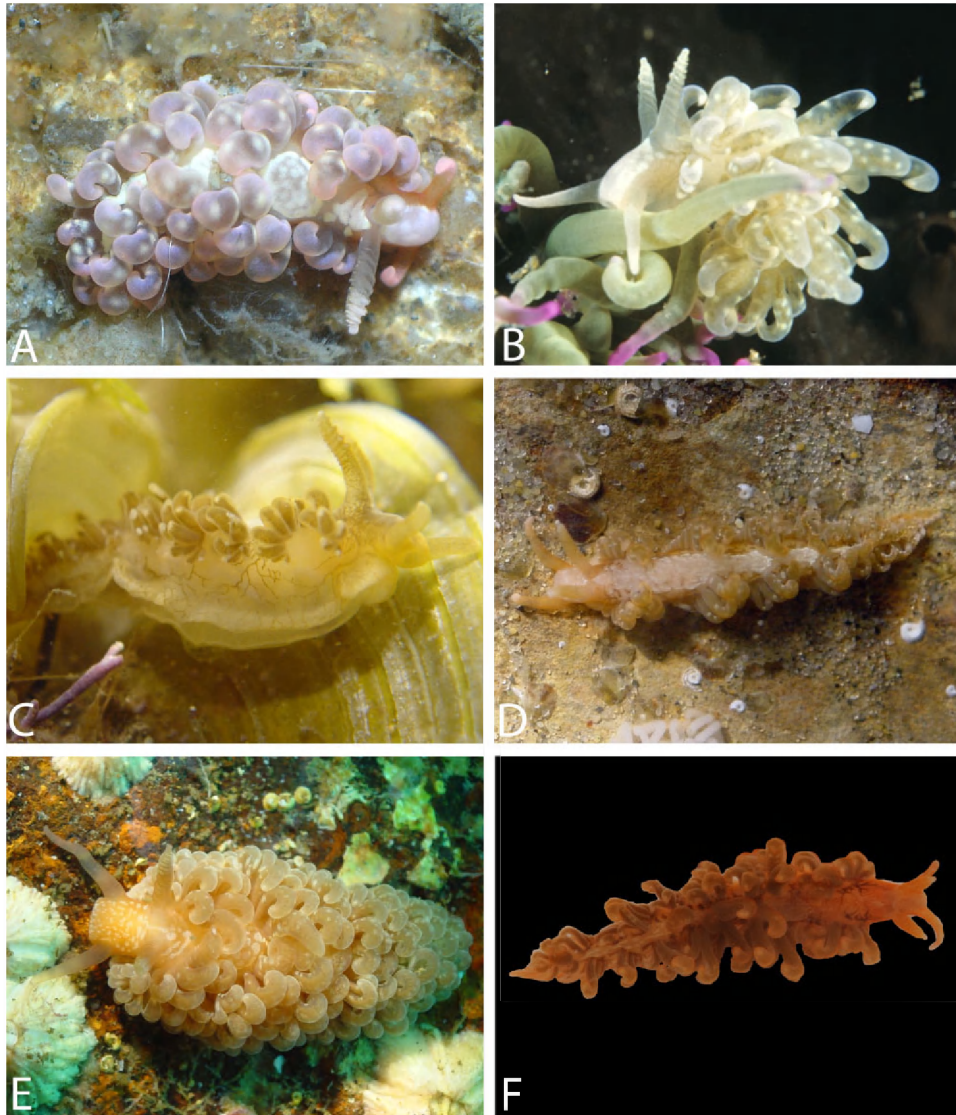
*Eolis conspersa* Fischer, 1869: 7.

?*Spurilla mograbina* Pruvot-Fol, 1953: 52, pl. I, fig. 12, pl. II, fig. 27.

*Spurilla vayssierei* García-Gómez & Cervera, 1985: 154.

#### Material examined

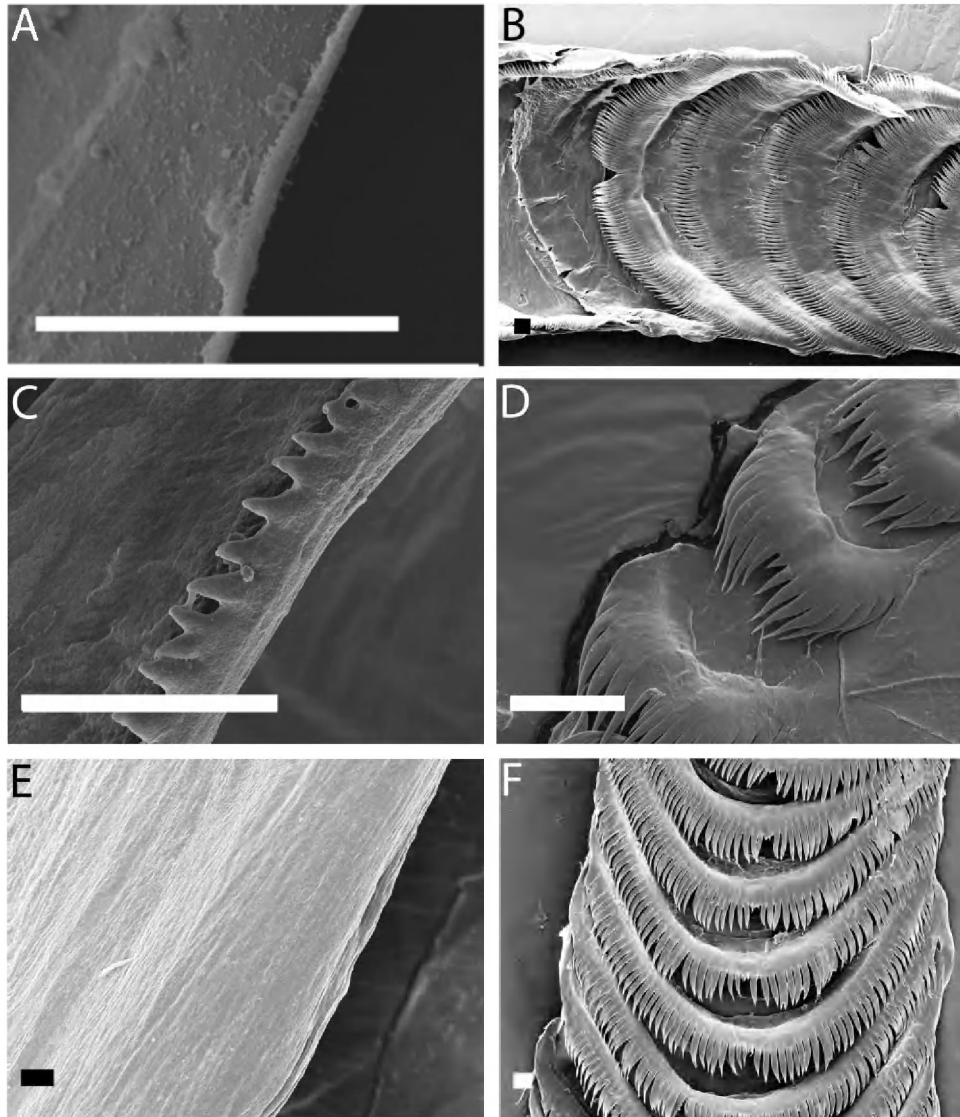
MNCN 15.05/63451, one specimen, dissected, 25 mm in length preserved, Spain, Huelva, El Portil, collected by Sergio García-Gómez, 09.iv.09; MNCN 15.05/63452, one specimen, dissected, 23 mm in length preserved, Spain, Huelva, El Portil, collected by Sergio García-Gómez, 09.iv.09; MNCN 15.05/63453, one specimen, dissected, 25 mm in length preserved, Balearic Is., Menorca, Cap Cavalleria, collected by Naoufal Tamsouri, 25.v.08; MNCN 15.05/63454, one specimen, dissected, 20 mm in length preserved, Balearic Is., Menorca, Cap Cavalleria, collected by Naoufal Tamsouri, 25.v.08; MNCN 15.05/63455, one specimen, dissected, 12 mm in length preserved, Spain, Cádiz, Santa María, collected by J. Lucas Cervera, 14.xii.94; MNCN 15.05/63465, one specimen, 10 mm in length preserved, Spain, Huelva, El Portil, collected by Sergio García-Gómez, 09.iv.12; LACM 178563, one specimen, dissected, 10 mm in length preserved, North of Spain, collected by Ismael Mata Valdés, 07.viii.12; CASIZ 175756, one specimen, dissected, 14 mm in length preserved, Portugal, Algarve, Ponta da Baleeira, collected by Yolanda



**Figure 1.** Different colour types of *Spurilla neapolitana*. A, specimen from France, Cap Ferret, Hortense Sud, photo by Marina Poddubetskaia MNCN 15.05/63467; B, specimen from Gulf of Naples, photo by Guido Villani, MNCN 15.05/63466; C, specimen from France, Cap Ferret, photo by Marina Poddubetskaia, CASIZ 192384; D, specimen from France, Cap Ferret, photo by Marina Poddubetskaia, MNCN 15.05/63463; E, specimen from Senegal, photo by Marina Poddubetskaia, MNCN 15.05/63456; F, specimen from Southern Spain, Huelva, El Portil, photo by Sergio García-Gómez, MNCN 15.05/63465.

Camacho-García, 08.vii.02; CASIZ 072582, one specimen, dissected, 20 mm in length preserved, Azores, Ilha São Miguel, Ilheu de Vila Franca, collected by Terrence M. Gosliner, 14.vii.88; MNCN 15.05/63463, one specimen, dissected, 13 mm in length alive, France, Cap Ferret, Grand Piquey, collected by Marina Poddubetskaia, 18.ix.04; MNCN 15.05/63464, one specimen, dissected, 22 mm in length alive, France, Cap Ferret, Grand Piquey, collected by Marina Poddubetskaia, 18.ix.04; MNCN 15.05/63467, one specimen, 10 mm in length alive, France, Cap

Ferret, Hortense Sud, collected by Marina Poddubetskaia, 09.ix.12; CASIZ 192384, one specimen, 13 mm in length preserved, France, Cap Ferret, collected by Marina Poddubetskaia, 18.iv.09; MNCN 15.05/63458, one specimen, dissected, 24 mm in length preserved, Italy, Gulf of Naples, collected by Guido Villani, 17.iii.09; MNCN 15.05/63459, one specimen, dissected, 20 mm in length preserved, Italy, Gulf of Naples, collected by Guido Villani, 17.iii.09; MNCN 15.05/63466, one specimen, 10 mm in length preserved, Italy, Gulf of Naples, collected by Guido



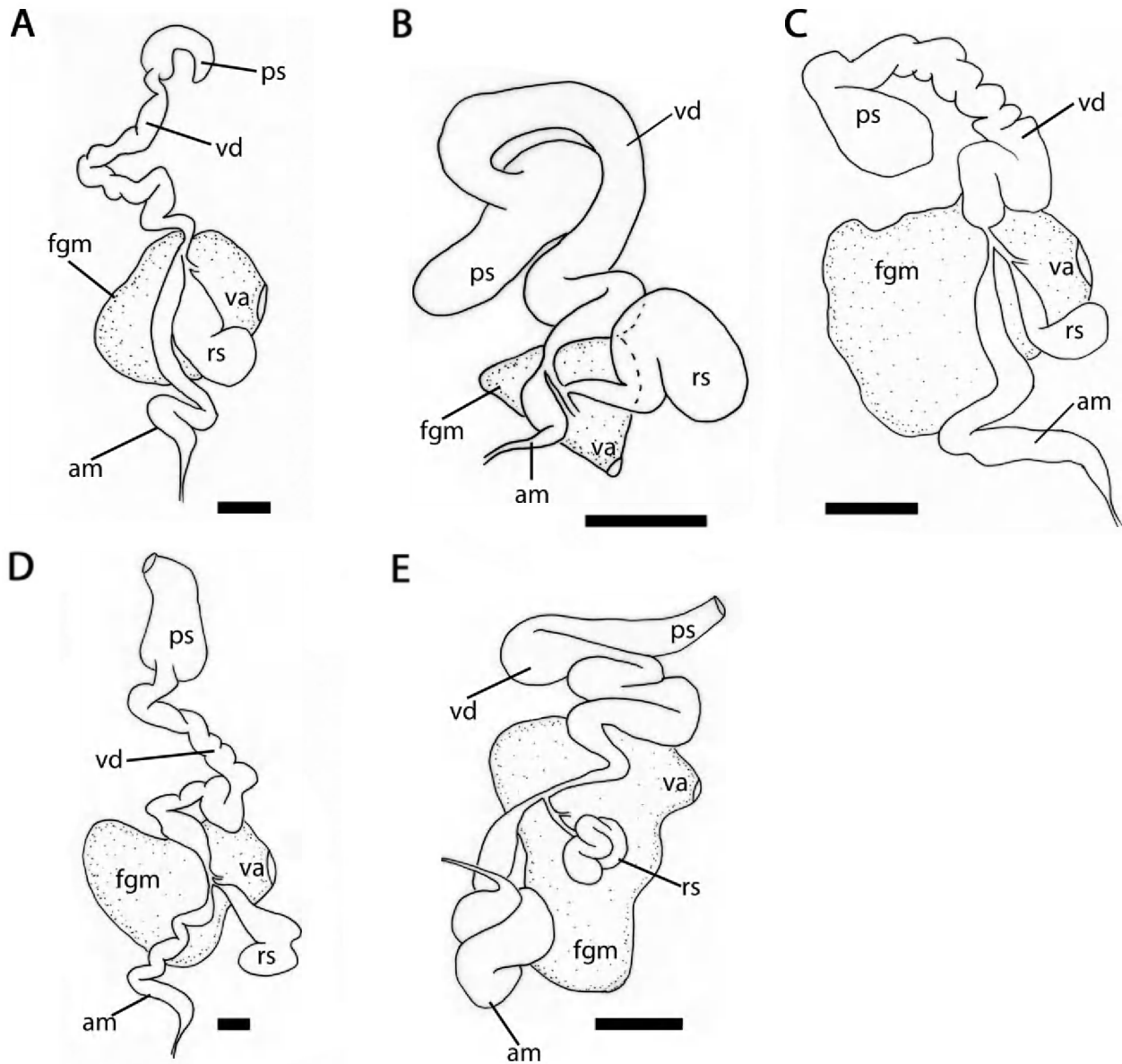
**Figure 2.** Scanning electron photographs. A, B, *Spurilla neapolitana*: A, detailed view of the masticatory border (MNCN 15.05/63463), specimen from France, Cape Ferret, scale bar = 100  $\mu$ m; B, radular teeth (CASIZ 175756), specimen from Portugal, Algarve, Ponta da Baleeira, scale bar = 20  $\mu$ m. C, D, *Spurilla sargassicola* (CASIZ 192385), specimen from Bahamas, Abaco: C, detailed view of the masticatory border, scale bar = 10  $\mu$ m; D, radular teeth, scale bar = 20  $\mu$ m. E, F, *Spurilla braziliana* (CASIZ 175737), specimen from Costa Rica, Pacific Coast, Guanacaste, Punta Carbón: E, detailed view of the masticatory border, scale bar = 10  $\mu$ m; F, radular teeth, scale bar = 20  $\mu$ m.

Villani, 17.iii.09; MNCN 15.05/63461, one specimen, dissected, 9 mm in length preserved, Croatia, Catoro, collected by Tancredi D'Onofrio, 09.xii.07; MNCN 15.05/63462, one specimen, dissected, 12 mm in length preserved, Croatia, Catoro, collected by Tancredi D'Onofrio, 09.xxii.07; MNCN 15.05/63460, one specimen, dissected, 15 mm in length preserved, Morocco, Agadir, Sidi Boufdail, collected by Naoufal Tamsouri, 10.iv.09; MNCN 15.05/63456, one specimen, dissected, 18 mm in length preserved, Senegal, collected by Marina Poddubetskaia, 08.vi.05;

MNCN 15.05/63457, one specimen, dissected, 24 mm in length preserved, Senegal, collected by Marina Poddubetskaia, 08.vi.05;

#### *Geographical distribution*

Originally described from the Mediterranean Sea (Delle Chiaje, 1841), this species has been reported from Israel (Schlesinger *et al.*, 2009) to Málaga, Spain (Luque, 1983, 1986), including the Balearic Islands (Ballesteros, Álvarez & Mateo, 1986). Within the eastern Atlantic, its geographic distribution ranges



**Figure 3.** Reproductive system: A, *Spurilla neapolitana*, specimen from Azores (CASIZ 072582), scale bar = 1 mm; B, *Spurilla sargassicola*, specimen from the Bahamas (MNCN 15.05/63468), scale bar = 0.5 mm; C, *Spurilla braziliana*, specimen from Brazil (MNCN 15.05/63471); D, *Spurilla onubensis* sp. nov., holotype (MNCN 15.05/60081), specimen from Spain, Huelva, scale bar = 0.5 mm; E, *Spurilla dupontae* sp. nov., holotype (LACM 3254), specimen from the Bahamas, Stocking Island, scale bar = 1 mm. Abbreviations: am, ampulla; fgm, female gland mass; ps, penial sac; rs, receptaculum seminis; va, vagina; vd, vas deferens.

from Arcachon (France) (Bouchet & Tardy, 1976) to Cape Verde (Ev. Marcus & Er. Marcus, 1962), including the island of Madeira (Wirtz, 1999), the Azores (Wirtz, 1998; Malaquias, 2001), Tarifa, Spain (García-Gómez & Cervera, 1985), the Canary Islands (Ev. Marcus & Er. Marcus, 1962; Ortea *et al.*, 2001; Wirtz & Debelius, 2003), Morocco (present study), and Senegal (present study).

#### *External morphology (Fig. 1A–F)*

The body is elongate and moderately broad, tapering gradually to the tail. The anterior foot corners extend into tentaculiform processes. The body colour is highly variable: it ranges from translucent (Fig. 1A–E) to bright orange (Fig. 1F). White spots are present all over the dorsum. Depending on the specimen, these white spots can be more or less conspicuous. In some

areas, mainly over the head and the pericardium, the white spots can form a somewhat continuous patch. Some specimens also have a fine pattern of white pigmentation that covers the whole notum. Because of the presence of symbiotic zooxanthellae, a dark brown or dull olive green pattern of reticulations is easily distinguishable in most of the specimens, going from the head (including rhinophores), where it is more evident, and including the foot edges, to the posterior end of the body.

The rhinophores, the oral tentacles, and the foot corners are coloured similarly to the background of the animal. The rhinophores are perfoliate, bearing up to 25 lamellae. They are somewhat shorter than the oral tentacles. All the specimens have rhinophores with white apices, but some of them also have white or cream pigment on the upper third of the rhinophores. The coloration of the tips of the oral tentacles is usually lighter than the background coloration. Some specimens, usually those with translucent body colour, have pinkish tips.

The cerata length is variable, but all are long and thick, narrower towards the tip, which curves inwards. The cerata extend from behind the rhinophores to the posterior end of the body, and are arranged in up to ten arches, leaving a distinct gap between pre and post-pericardial groups. Each arch contains between four and 39 cerata, decreasing in size towards the foot. The cerata are translucent, so the brownish green or greyish ramifications of the digestive gland are visible throughout the ceratal wall. The apices of the cerata are translucent white. The ceratal surface has white marks, although their density and size may vary. Some specimens also have purplish and/or pinkish pigment covering the whole surface of the cerata. The anus is cleioproctic, located within the second right ceratal arch. The genital opening is located among the cerata of the anteriormost group on the right.

#### Anatomy

The masticatory border of the jaws is finely denticulate (Fig. 2A). The radular formulae are  $16 \times 0.1.0$  (MNCN 15.05/63463, 13 mm; MNCN 15.05/63464, 22 mm) and  $27 \times 0.1.0$  (CASIZ 175756, 14 mm). The teeth are progressively smaller towards the posterior region of the radula. The radular teeth are biarched, with 24–60 elongate, fine, and acutely pointed denticles on either side of the small and triangular central cusp (Fig. 2B). The large, composite and conspicuous oral glands lie dorsolaterally to the buccal bulb. Salivary glands are absent.

The reproductive system is dialucic (Fig. 3A). The preampullary duct widens into the elongate ampulla. The latter narrows again before dividing into the oviduct and vas deferens. The elongate and thin

vas deferens enters the wider proximal portion of the penial sac, which contains the unarmed penial papilla. The oviduct is short and connects to the moderately large receptaculum seminis. The remaining portion of the oviduct departs from the base of the receptaculum and enters the female gland. The vagina opens ventral to the penis.

#### Remarks

*Eolis neapolitana* was first illustrated by Delle Chiaje in 1841, but the description was not published until 3 years later in an appendix treating the genus *Eolis* (Delle Chiaje, 1844: vol. 8, p. 7). Some authors (e.g. García-Gómez & Cervera, 1985) have given 1823 as the original date of publication; however, this species does not appear in the first edition of Delle Chiaje's memoirs (1823–1830).

According to the literature, *S. neapolitana* is a nearly circumtropical species and displays a broad range of colour variation (e.g. Rudman, 1999 and Gosliner, Behrens & Valdés, 2008); however, our molecular data reveal the existence of several distinct species among the specimens assigned to this species. These results indicate that the geographical distribution of *S. neapolitana* is restricted to the Eastern Atlantic and Mediterranean. Despite this proposed reduction of its geographic and coloration ranges, *S. neapolitana* still covers a large portion of the north-eastern Atlantic coast, from Cap Ferret (France) to Cape Verde, and varies in body colour from translucent to bright orange (Fig. 1).

The extensive colour variation of *S. neapolitana* is attributed to its varied diet rather than the geographical distribution of individuals (Haefelfinger, 1969; Uribe & Pacheco, 2012). Another hypothesis is that this variability is associated with an ontogenetic change, as the paler morphotypes are usually the smaller specimens (less than 10 mm), whereas larger specimens usually have a darker coloration, mainly orange (L. Carmona, L. R. Bonnie, M. Pola, T. M. Gosliner, Á. Valdés & J. L. Cervera, pers. observ.). The intraspecific variation of the masticatory border of this species is widely accepted (Gosliner, 1980; García-Gómez & Cervera, 1985). In fact, our specimens have both types of masticatory edge: minutely denticulate (Fig. 2A) and smooth (not shown). Although we could not find any ontogenetic variation pattern, the masticatory border without denticles is the most common in our specimens. García-Gómez & Cervera (1985) conducted the last published revision of the true *S. neapolitana*. Even though they examined a large number of specimens, they did not find any with a denticulate masticatory border. They proposed a new species, *S. vayssierei*, for specimens of *Spurilla* attributed to *S. neapolitana* with denticulate masticatory borders. Our results



reject the validity of *S. vayssierei* because the ornamentation of the masticatory edge cannot be diagnostic in *Spurilla*, as a result of its intraspecific variation. Hence, we conclude that *S. vayssierei* is a junior synonym of *S. neapolitana*.

*Spurilla mograbina*, from Morocco, was described and illustrated by Pruvot-Fol (1953). The illustration clearly depicts a *Spurilla* species with perfoliate rhinophores, cerata curved inwards, and white and dull pink spots over the pinkish dorsum (Pruvot-Fol, 1953: plate I, fig. 12); however, this colour pattern does not match any of the colour variations observed so far by us in *S. neapolitana* (Fig. 1A–F). Thus, with the available information it is not possible to determine the validity of this species with certainty; however, because the type locality of *S. mograbina* is well within the range of *S. neapolitana*, and both species are similar morphologically, we render *S. mograbina* as a possible junior synonym of *S. neapolitana* until more specimens become available.

*SPURILLA SARGASSICOLA* BERGH, 1871

(FIGS 2C–D, 3B, 4A–B)

*Spurilla sargassicola* Bergh, 1871: 161, pl. XIII, figs 9–19.

*Spurilla* sp. Redfern, 2001: 181, pl. 121, fig. 746A,B.

*Material examined*

CASIZ 184523, one specimen, dissected, 4 mm in length preserved, Bahamas, Abaco, collected by Colin Redfern, 19.ii.04; MNCN 15.05/63468, one specimen, dissected, 4 mm in length preserved, Bahamas, Abaco, collected by Colin Redfern, 16.ix.04; CASIZ 192385, one specimen, dissected, 2 mm in length preserved, Bahamas, Abaco, collected by Colin Redfern, 17.ix.03; CPIC 00815, one specimen, dissected, 11 mm in length preserved, Stocking Island, Bahamas, collected by Anne DuPont, 28.ii.09; CPIC 00816, one specimen, dissected, 15 mm in length preserved, Bahamas, Stocking Island, collected by Anne DuPont, 28.ii.09; CPIC 00817, one specimen, dissected, 11 mm in length preserved, Bahamas, Stocking Island, collected by Anne DuPont, 28.ii.09.

*Geographical distribution*

Originally described from the Sargasso Sea (Bergh, 1861; Redfern, 2001; Valdés *et al.*, 2006), this species is also found in the Bahamas (Valdés *et al.*, 2006) and in the National Park Arrecife Alacranes (Mexico) (Sanvicente-Añorve *et al.*, 2012).

*External morphology (Fig. 4A–B)*

The body is elongate and slender, tapering gradually to the posterior end. The anterior foot corners extend

into tentaculiform processes. The body colour is translucent pale brown, with a light ochre, greenish, or brownish reticulation pattern all over it. Because of the presence of the zooxanthellae, this pattern is more evident near the head and on the foot edges. Cream white spots are found all over the dorsum (from the head to the posterior end). Depending on the specimen, these spots can form a continuous patch, but never cover the sides of the animal, which are dark brown or dark ochre.

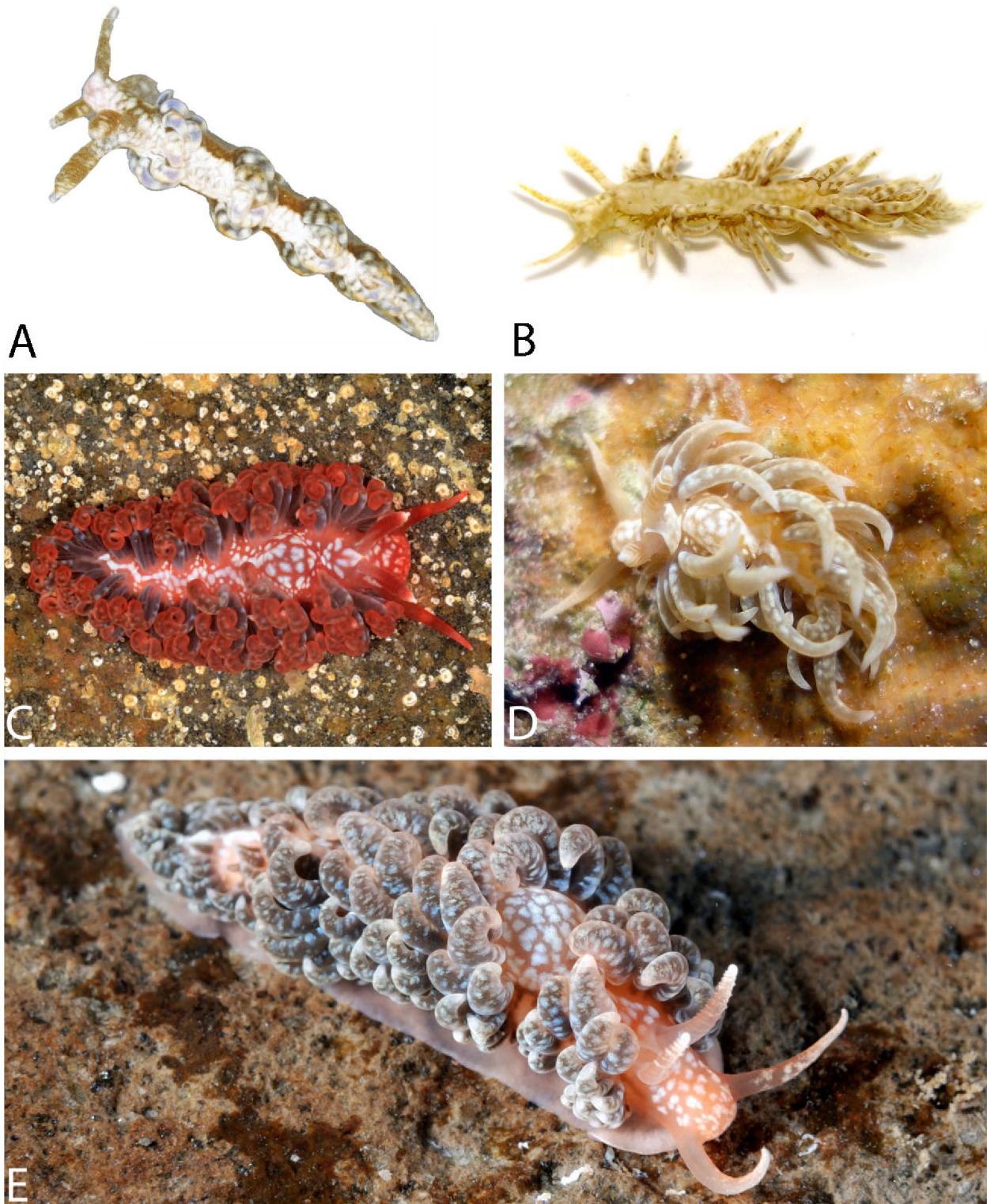
The coloration of the rhinophores ranges from pale cream to dark brown. They are somewhat shorter than the oral tentacles. The rhinophores are perfoliate, bearing between six and nine lamellae. The rhinophores of all the specimens have white apices, but some also have white or cream pigment on the upper third of the rhinophores, including the lamellae. The oral tentacles and the foot corners are the same colour as the body background. Some specimens have cream white marks on the oral tentacles.

The cerata length is variable, but they are usually short and thick, narrower towards the tip, which is curved inwards. Some other specimens have long and thin cerata. The cerata extend from behind the rhinophores to the posterior end of the body, and are arranged in up to seven arches, leaving a distinct gap between them. Each arch contains between three and seven cerata, decreasing in size towards the foot. The apices of the cerata are translucent white, and all the specimens have white marks all over the ceratal surface, although their density and size vary. Some specimens also have purplish and/or ochre pigment among the white spots. The cleioproctoc anus is placed within the second right arch. The genital opening is located within the cerata of the anteriormost group on the right.

*Anatomy*

The masticatory border of the jaws is denticulate (Fig. 2C). The radular formulae are  $10 \times 0.1.0$  (MNCN 15.05/63468, 4 mm) and  $18 \times 0.1.0$  (CASIZ 184523, 4 mm). The teeth are progressively smaller in the posterior region of the radula. The radular teeth are biarched, with between eight and 14 moderately broad and acutely pointed denticles on either side of the small and triangular central cusp (Fig. 2D). The large, composite, and conspicuous oral glands lie dorsolaterally to the buccal bulb. Salivary glands are absent.

The reproductive system is diaulic (Fig. 3B). The preampullary duct widens into the short ampulla that narrows again before dividing into the oviduct and vas deferens. The conspicuous vas deferens enters the wider proximal portion of the penial sac, which contains the unarmed penial papilla. The short oviduct connects to a large receptaculum seminis. The



**Figure 4.** Photographs of living animals. A, B, different morphotypes of *Spurilla sargassicola*; A, specimen from the Bahamas, Abaco, photo by Colin Redferns; B, specimen from the Bahamas, Stocking Island, photo by Anne DuPont (CPIC 00816). C–E, different morphotypes of *Spurilla braziliana*; C, adult from Brazil, photo by Carlos Cunha, MZUSP 103232; D, juvenile from Brazil, photo by Carlos Cunha, MZUSP 103196; E, most common morphotype in the Pacific, specimen from Japan, photo by Rie Nakano.

remaining portion of the oviduct departs from the base of the receptaculum and enters the female gland. The vagina opens ventral to the penis.

#### Remarks

Some authors (e.g. Sanvicente-Añorve *et al.*, 2012; Gofas, 2013) considered Bergh (1861) as the original date of publication of *S. sargassicola*; however, Bergh (1861) did not provide a description of the animals, instead he provided descriptions of the ingested nematocysts. Thus, Bergh's (1861) descriptions do not meet the criteria for a valid species description under Article 12 of the Code of Zoological Nomenclature (ICZN, 1999), as no descriptions or illustrations of the actual animals, or indications, are provided. Bergh described *S. sargassicola* properly 10 years later (Bergh, 1871); therefore, we consider Bergh (1871) as the real date of publication, and state the name proposed by Bergh (1861), *Facelina? sargassicola*, as *nomen nudum*. On the other hand, Dautzenberg & Durouchoux (1906) reported *S. sargassicola* (under the name of *Eolis sargassicola*) from Saint-Malo, France. The specimens were described as large (up to 55 mm), with red rhinophores and oral tentacles. Hence, it seems evident that Dautzenberg & Durouchoux (1906) did not find *S. sargassicola*, as this species is mainly white and is restricted to the western Atlantic.

*Spurilla sargassicola* has been reported more often as a junior synonym of *S. neapolitana* than as a valid species; however, recent papers have challenged this view, and its status as a different species from *S. neapolitana* has become more widely accepted in recent years (Valdés *et al.*, 2006; Sanvicente-Añorve *et al.*, 2012; Carmona *et al.*, 2013). Our molecular results clearly separate *S. sargassicola* and *S. neapolitana* into two allopatric species. *Spurilla sargassicola* differs from *S. neapolitana* because of its predominately white coloration and its conspicuous gap between each ceratal group. Additionally, *S. sargassicola* also has distinct life-history characteristics, as it is usually found on free-living *Sargassum* and is the only pelagic species of Aeolidiidae. These morphological and ecological features also distinguish *S. sargassicola* from the remaining species of this genus.

As in the case of *S. neapolitana*, intraspecific variation of the masticatory edge was observed. Although Figure 2C shows somewhat conspicuous denticles over the masticatory border, some of our specimens have a smooth edge (not shown). As this variability is quite common within this genus (see *S. neapolitana* remarks), we do not consider this difference to be taxonomically significant.

This is the first publication that includes detailed descriptions of genitalia and oral glands of *S. sargassicola*.

#### *SPURILLA BRAZILIANA* MACFARLAND, 1909 (FIGS 2E–F, 3C, 4C–E)

*Spurilla braziliana* MacFarland, 1909: 91, pl. XVI, XVII, XVIII. and XIX, figs 83–96.

*Eolidina gabriellae* Vannucci, 1952: 284–288, figs 1–4.

*Spurilla neapolitana* var. *braziliana* Er. Marcus, 1955: 184, pl. 29, figs 270–274.

*Aeolidiella albopunctata* Lin, 1992: 184, figs 4–5.

#### Material examined

*Holotype*: CASIZ 019731, one specimen, 23 mm dissected, Brazil, Algoas, collected by MacFarland, 28.vii.1899.

*Other material*: CASIZ 175737, one specimen, dissected, 13 mm in length preserved, Costa Rica, Pacific Coast, Guanacaste, Punta Carbón, collected by Yolanda Camacho-García, 13.i.01; CASIZ 175735, one specimen, dissected, 6 mm in length preserved, Costa Rica, Pacific Coast, Guanacaste, Punta Carbón, collected by Yolanda Camacho-García, 11.i.01; CASIZ 172374, one specimen, dissected, 14 mm in length preserved, Panama, Pacific Coast, Golfo de Chiriqui, Isla Uva, collected by Mónica Medina, 16.ii.01; CASIZ 072111, one specimen, dissected, 25 mm in length preserved, Mexico, Baja California Sur, Gulf of California, collected by Terrence M. Gosliner, 14.i.84; MNCN 15.05/63471, one specimen, dissected, 10 mm in length preserved, Brazil, Cabo Frio, collected by Vinicius Padula, 16.viii.07; MNCN 15.05/63472, one specimen, dissected, 10 mm in length preserved, Cuba, Playa Girón, collected by J. Lucas Cervera, 16.vii.08; CPIC 00810, one specimen, dissected, 12 mm in length preserved, Peanut Island, Riviera Beach, Florida, collected by Anne DuPont, 08.xi.07; CPIC 00811, one specimen, dissected, 11 mm in length preserved, Peanut Island, Riviera Beach, Florida, collected by Anne DuPont, 08.xi.07; CPIC 00813, one specimen, dissected, 7 mm in length preserved, Peanut Island, Riviera Beach, Florida, collected by Anne DuPont, 08.xi.07; CASIZ 076124, one specimen, dissected, 22 mm in length preserved, Hawaii, Oahu, Kewalo Basin, collected by Terrence M. Gosliner, xi.72; CASIZ 187750, one specimen, dissected, 7 mm in length preserved, Japan, Honshu, Shizuoka Prefecture, Ose cape, collected by Rie Nakano, 07.vii.11.

#### Geographical distribution

Originally described from Alagoas, Brazil (MacFarland, 1909), this species has been reported (under the name of *S. neapolitana*) from the western Atlantic Coast: the Caribbean Sea (Valdés *et al.*, 2006), Florida, Mexico, Colombia, Cuba (the present

study), Jamaica (Miloslavich *et al.*, 2010), Puerto Rico (Miloslavich *et al.*, 2010), and in the northern part of the Magellanic Province (Forcelli, 2000). It has also been reported from the Pacific: Hawaiian Islands (Kay, 1979; Gosliner, 1980; Pittman & Fiene, 2013), the Pacific coast of Costa Rica (Camacho-García *et al.*, 2005), Peru (Uribe & Pacheco, 2012), Japan (Hamatani, 2000), China (Lin, 1992), and Australia (Willan, 2006).

#### *External morphology (Fig. 4C–E)*

The body is elongate and moderately broad, tapering gradually to the posterior end. The anterior foot corners extend into tentaculiform processes. The body colour is variable. This variability results from ontogenetic changes (Domínguez *et al.*, 2008). The youngest individuals are cream white (Fig. 4D), whereas the adults have a coloration that ranges from pale orange to vermillion red (Fig. 4C, E). White spots occur all over the dorsum (from the head to the posterior end) in both juveniles and adults. Depending on the specimen, these white spots can be more or less conspicuous. Right behind the rhinophores and behind the pericardium, the white spots can form a confluent patch. A reticulate pattern produced by the presence of zooxanthellae is usually absent, or at least not easily distinguishable, but when present it is restricted to both sides of the head, just below the rhinophores, as a short branch (Fig. 4E), as well as on the insertions of the cerata (Fig. 4D–E).

The rhinophores, the oral tentacles, and the foot corners have the same colour as the rest of the body. The rhinophores are perfoliate, bearing 11–14 lamellae with a white apex. They are somewhat shorter than the oral tentacles. The coloration of the tips of the oral tentacles is lighter than the background.

The cerata length varies depending on the size of the animals. Adults have short and thick cerata, and their tips are curved inwards. The cerata of the juveniles are long, thin, and slightly curved inwards (Fig. 4D). The cerata are arranged in up to nine arches, leaving a distinct gap between pre- and post-pericardial groups. They extend from behind the rhinophores to the posterior end of the body. Each arch contains between five and 26 cerata, decreasing in size towards the foot. The apices of the cerata are translucent white. The cerata are translucent, light brown (juveniles), or bluish dark grey or dark green (adults), with the ramifications of the digestive gland visible through the ceratal wall. All the specimens have white markings all over the ceratal surfaces, although their density and size vary. Only the vermillion red specimens have a vermillion red band on the distal third of the cerata. The anus is cleioproctic and is located within the second right

ceratal arch. The genital opening is located within the cerata of the anteriormost group on the right.

#### *Anatomy*

The masticatory border is smooth (Fig. 2E). The radular formulae are  $9 \times 0.1.0$  (CPIC 00811, 11 mm),  $12 \times 0.1.0$  (CASIZ 175735, 6 mm; CASIZ 175737, 13 mm),  $16 \times 0.1.0$  (CPIC 00810, 12 mm), and  $28 \times 0.1.0$  (MNCN 15.05/63472, 10 mm). The teeth are progressively smaller towards the posterior region of the radula. The radular teeth are biarched, with 20–55 elongate, moderately broad, and acutely pointed denticles on either side of the triangular central cusp (Fig. 2F). The large, composite, and conspicuous oral glands lie dorsolaterally to the buccal bulb. Salivary glands are absent.

The reproductive system is diaulic (Fig. 3C). The preampullary duct widens into the elongate ampulla that narrows again before dividing into the oviduct and vas deferens. The conspicuous vas deferens enters the wider proximal portion of the penial sac, which contains the unarmed penial papilla. The oviduct is short and connects to the receptaculum seminis. The remaining portion of the oviduct departs from the base of the receptaculum and enters the female gland. The vagina opens ventral to the penis.

#### *Remarks*

MacFarland (1909) erected the species name *S. braziliiana* based on a single preserved specimen collected in Brazil. Despite the fact that the original colour of the holotype had entirely disappeared before it was examined, MacFarland (1909) determined that 'general differences shown by this specimen in anatomical organization, especially as shown by the mandibles and radula, seem sufficient to authorize its recognition as a member of a species distinct from the European ones already described'. Many authors have rendered *S. braziliiana* as junior synonym or a variety of *S. neapolitana* (Engel, 1925; Er. Marcus, 1955, 1957; Just & Edmunds, 1985; Domínguez *et al.*, 2008; García-García *et al.*, 2008). Other authors pointed out the possibility that the western Atlantic *Spurilla* could be a distinct species from the Mediterranean *S. neapolitana* (Forcelli, 2000; Schrödl, 2003). Carmona *et al.* (2013) recognized the western Atlantic specimens attributed to *S. neapolitana* as *S. braziliiana*. Our molecular results agree with Carmona *et al.* (2013) and confirm the presence of this species in the Pacific. Regarding the morphological features of this species, it shows ontogenetic variation in coloration (Domínguez *et al.*, 2008). Excluding the vermillion red colour forms of *S. braziliiana*, the latter species and *S. neapolitana* have very similar colour patterns; however, the conspicuous branches of the digestive gland normally visible in *S. neapolitana*

(Fig. 1B–D, F) are not easily recognizable in *S. braziliana*, and if present are restricted to a small branch laterally on the head (Fig. 4E), and/or a small and a short branch on the ceratal insertions (Fig. 4D–E). Internally, MacFarland (1909) pointed out that the jaws of *S. braziliana* were noticeably smaller than those of *S. neapolitana*. After comparing several specimens from São Paulo (Brazil), Er. Marcus (1955) concluded that the only difference between these two species was the smaller jaws in *S. braziliana*; however, we have not been able to confirm this difference. Instead, we have noticed that the denticles of the radular teeth of *S. neapolitana* are usually thinner compared with those of *S. braziliana* (Fig. 2A, E). Nevertheless, both species have too much intraspecific variation to determine whether this difference can be considered taxonomically significant.

Carmona *et al.* (2013) indicated that *S. braziliana* was the only species of *Spurilla* found in the Pacific Ocean. In this study we have included several specimens collected from Japan, Australia, and the Pacific coast of Costa Rica, confirming the previous hypothesis and conclusively extending the known distribution of *S. braziliana* into the Pacific Ocean. Pacific specimens seem to have a more limited colour range: mainly orange, with conspicuous white spots over the dorsum and dark greenish grey cerata, with the same marks as the rest of the body (Fig. 4E). The ontogenetic variation shown in the Atlantic population has not been found in the Pacific specimens so far.

Vannucci (1952) described the aeolidiid species *Eolidina (Spurilla) gabriellae* from Brazil. This author observed the animals alive, and illustrated two specimens with a bright orange foot, translucent cerata, and dark brown digestive gland. Vannucci (1952) pointed out that the cerata of this new species were arranged in arches and rows, but no known species of *Spurilla* has such a ceratal arrangement. Thus, we suspect that this difference is probably caused by the preservation of the specimens. Vannucci (1952) also recognized *S. braziliana* as the closest relative of *S. gabriellae*, but based on differences in body size, ceratal arrangement, and radular and jaw morphology, he considered *S. gabriellae* as distinct species. This species, however, has been regarded as a junior synonym of *S. neapolitana* (Schmekel & Portmann, 1982; Domínguez *et al.*, 2008; García-García *et al.*, 2008). We conclude that the morphological differences indicated by Vannucci (1952) are not taxonomically significant, and therefore *S. gabriellae* is a junior synonym of *S. braziliana*.

Lin (1992) described the new species *Aeolidiella albopunctata* based on specimens collected from Hong Kong. Lin (1992) described this species as orange

reddish, with orange–yellow oral tentacles, a green–brown digestive gland, and large opaque white spots over the dorsum. According to Lin (1992), *A. albopunctata* also has small white flecks over the head, the oral tentacles, the rhinophores, and the cerata. Lin (1992) illustrated the living animal, the radular tooth, and the masticatory border. This author pointed out that the cerata of *A. albopunctata* were arranged in rows and the rhinophores had ‘longitudinal folds above’. Based on the comparison of the external coloration and the radular teeth of *A. albopunctata* with *S. braziliana*, and taking into account that the geographical range of *S. braziliana* includes the Pacific Ocean, we consider *A. albopunctata* to be a junior synonym of *S. braziliana*. Differences in the ceratal arrangement and the ornamentation of the rhinophores seem to be caused by the preservation of the specimens, and therefore they are not taxonomically significant.

#### ***SPURILLA ONUBENSIS* SP. NOV.**

(FIGS 3D, 5A, 6A–B)

*Spurilla* sp. A: Just & Edmunds (1985: 144, pl. 68, figs A–D).

*Spurilla* sp. A: Carmona *et al.* (2013: 6).

#### *Material examined*

*Holotype*: MNCN 15.05/60081, one specimen, dissected, 15 mm in length preserved, Spain, Huelva, El Portil, collected by Sergio García-Gómez, iv.09, deposited at MNCN.

*Paratype*: MNCN 15.05/60082, one specimen, dissected, 18 mm in length preserved, Spain, Huelva, El Portil, collected by Sergio García-Gómez, iv.09, deposited at the MNCN.

*More material*: MNCN 15.05/66445, one specimen, dissected, 15 mm in length preserved, Morocco, David (Atlantic Ocean), collected by Naoufal Tamsouri, 23.vii.09.

#### *Type locality and habitat*

Spain, Huelva, El Portil. Found in intertidal area, in tide pools and under the rocks.

#### *Geographical distribution*

So far, this species is only known from Arcachon (France) (Just & Edmunds, 1985), Huelva (south-eastern Spain; present study), and David (Morocco, Atlantic Ocean; present study).

#### *Etymology*

The Latin word *onubensis* refers to the Spanish Province of Huelva (Onuba during Roman times),



**Figure 5.** Photographs of living animals. A, *Spurilla onubensis* sp. nov. specimen from Southern Spain, Huelva, photo by Sergio García-Gómez, (MNCN 15.05/60081); B, *Spurilla dupontae* sp. nov. specimen from the Bahamas, Stocking Island, photo by Anne DuPont (LACM 3254).

which is the hometown of Sergio García-Gómez who kindly provided us with numerous specimens of Aeolidiidae from this area, including material of this new species.

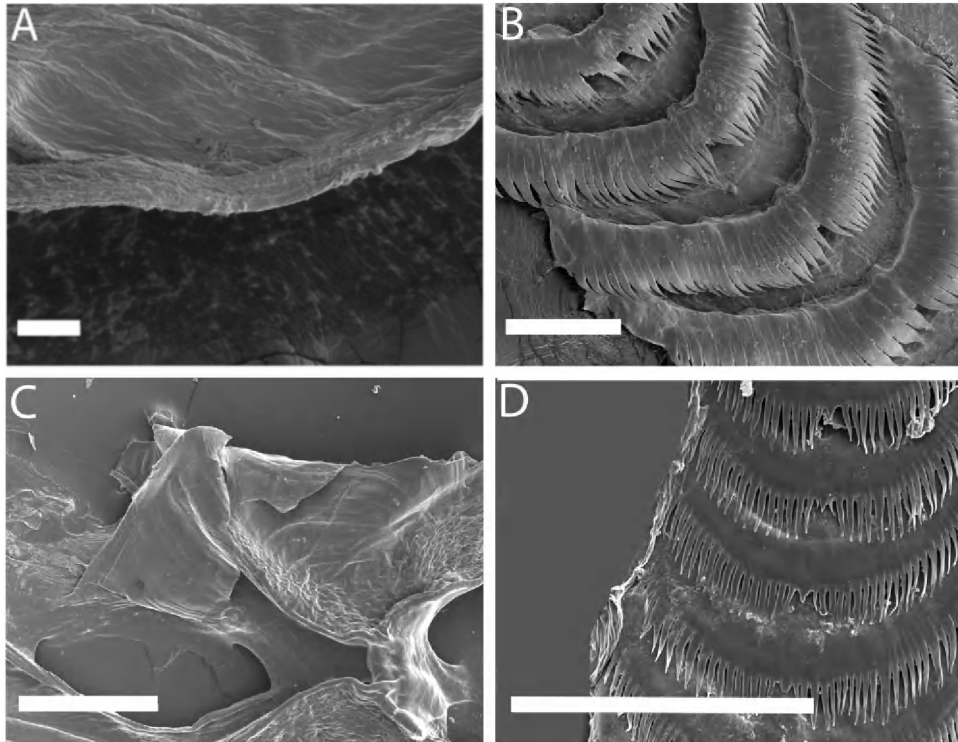
*External morphology (Fig. 5A)*

The body is elongate and moderately broad, tapering gradually to the posterior end. The anterior foot corners extend into tentaculiform processes. The body colour is translucent bright orange. The notum is devoid of any kind of spots, marks, or pigment. The branches of the digestive gland form a reticulate pattern. The latter is restricted to both sides of the head, just below the rhinophores, and forms short branches (Fig. 5A).

The rhinophores, the oral tentacles, and the foot corners have the same colour as the rest of the body.

The rhinophores are perfoliate, bearing around ten lamellae, and have white apices. They are slightly shorter than the oral tentacles. The coloration of the tips of the oral tentacles is lighter than the background colour of the animals.

The ceratal length is rather uniform. The cerata are short and thick, with their tips curved inwards. The cerata are arranged in up to 11 arches, leaving a distinct gap between the pre- and post-pericardial groups. The cerata extend from behind the rhinophores to the tail. Each arch contains between two and 27 cerata, decreasing in size towards the foot. The cerata are translucent orange, with translucent white apices. The dark olive green ramifications of the digestive gland are visible through the ceratal wall. The cleioproctical anus is located within the second right arch. The genital aperture is located



**Figure 6.** Scanning electron photographs. A, B, *Spurilla onubensis* sp. nov. (MNCN 15.05/60082), specimen from Spain, Huelva; A, detailed view of the masticatory border, scale bar = 20  $\mu$ m; B, radular teeth, scale bar = 100  $\mu$ m. C, D, *Spurilla dupontae* sp. nov. (LACM 3254), specimen from the Bahamas, Stocking Island; C, jaws, scale bar = 500  $\mu$ m; D, radular teeth, scale bar = 200  $\mu$ m.

among the cerata of the most anterior group on the right.

#### Anatomy

The masticatory border is smooth (Fig. 6A). The radular formulae are  $17 \times 0.1.0$  (MNCN 15.05/60081, 15 mm),  $20 \times 0.1.0$  (MNCN 15.05/60082, 18 mm), and  $25 \times 0.1.0$  (MNCN 15.05/66445, 15 mm). The teeth are progressively smaller towards the posterior region of the radula. The radular teeth are biarched with 24–38 elongate, moderately broad, and acutely pointed denticles on either side of the triangular central cusp (Fig. 6B). The large, composite, and conspicuous oral glands lie dorsolaterally to the buccal bulb. Salivary glands are absent.

The reproductive system is diaulic (Fig. 3D). The preampullary duct widens into the ampulla that narrows again before dividing into the oviduct and vas deferens. The elongate and thin vas deferens enters the wider proximal portion of the penial sac, which contains the unarmed penial papilla. The short oviduct connects to the receptaculum seminis. The remaining portion of the oviduct departs from the base of the receptaculum and enters the female gland. The vagina opens ventral to the penis.

#### Remarks

Just & Edmunds (1985) compiled unpublished illustrations by Lemche, including one of a *Spurilla* species under the name *Spurilla* sp. A (illustrated in plate 68). This plate depicts a specimen with exactly the same colour pattern as our specimens of *S. onubensis* sp. nov. described herein. Both lack any trace of white spots, clearly visible on the notum and the cerata in other *Spurilla* species, and have a bright-orange body. Pruvot-Fol (1953) described *S. mograbina* from Temara (Morocco), which is close to David (the locality where one of our specimens of *S. onubensis* sp. nov. was collected), and illustrated a single specimen with opaque cream spots scattered on the dorsal side of the cerata and the notum. *Spurilla onubensis* sp. nov. is clearly different from *S. mograbina* as our specimens lack such pigmentation. Although our specimens were initially identified as *S. neapolitana* (see Carmona *et al.*, 2013), both morphological and molecular data clearly separate *Spurilla onubensis* sp. nov. from *S. neapolitana* and the remaining members of this genus.

To date, *S. onubensis* sp. nov. and *S. neapolitana* are the only eastern Atlantic species of this genus, but they were not recovered as sister species in the phylogenetic

analyses (see molecular results). Furthermore, the geographical range of *S. neapolitana* overlaps with that of *S. onubensis* sp. nov.

**SPURILLA DUPONTAE SP. NOV.**

(FIGS 3E, 5B, 6C–D)

*Material examined*

*Holotype*: LACM 3254, dissected, 38 mm in length preserved, Bahamas, Stocking Island, collected by Anne DuPont, 13.iii.08.

*Paratype*: LACM 3255, one specimen, dissected, 32 mm in length preserved, Stocking Island, Bahamas, collected by Anne DuPont, 13.iii.08.

*Type locality and habitat*

Bahamas. Found in a sheltered harbour, on *Padina* sp.

*Geographical distribution*

So far, only known from the Bahamas.

*Etymology*

This species is dedicated to Anne DuPont, who collected the first specimens of this species.

*External morphology (Fig. 5B)*

The body is elongate and slender, tapering gradually to the posterior end. The anterior foot corners extend into tentaculiform processes. The body colour is translucent pale brown overlaid with a conspicuous ochre or brownish reticulate pattern. Cream white spots are found all over the dorsum (from the head to the posterior end). Their density and size vary with the specimen.

The rhinophores, the oral tentacles, and the foot corners have the same colour as the rest of the body, with the brownish or ochre reticulate pattern. The rhinophores are perfoliate, bearing 16 lamellae, and are slightly shorter than the oral tentacles. The apices of the rhinophores are white. Some specimens have white-tipped oral tentacles.

The cerata are long, cylindrical, with round apices, and uniform in diameter throughout most of their length, with their tips curved inwards. They extend from behind the rhinophores to the posterior end. The cerata are arranged in up to seven arches of decreasing length towards the posterior end, with a distinct gap between them. Each arch contains between three and nine cerata, decreasing in size towards the foot. The ceratal apices are translucent white. White cream spots are present on the ceratal surface, although their density and size vary between individuals. The anus is cleioproctic and is located within the

second right arch. The genital opening is situated among the cerata of the anteriormost group on the right.

*Anatomy*

The masticatory border of the jaws is smooth (Fig. 6C). The radular formulae are  $10 \times 0.1.0$  (LACM 3255, 32 mm) and  $21 \times 0.1.0$  (LACM 3254, 38 mm). The teeth are progressively smaller in the posterior region of the radula. The radular teeth are biarched, with up to 29 acutely pointed denticles on either side of the small and triangular central cusp (Fig. 6D). Oral and salivary glands were not examined.

The reproductive system is diaulic (Fig. 3E). The preampullary duct widens into a conspicuous ampulla that narrows again before dividing into the oviduct and vas deferens. The moderately thick vas deferens enters the wider proximal portion of the penial sac, which contains the unarmed penial papilla. The short oviduct connects to a folded and convoluted receptaculum seminis. The remaining portion of the oviduct departs from the base of the receptaculum and enters the female gland. The vagina opens ventral to the penis.

*Remarks*

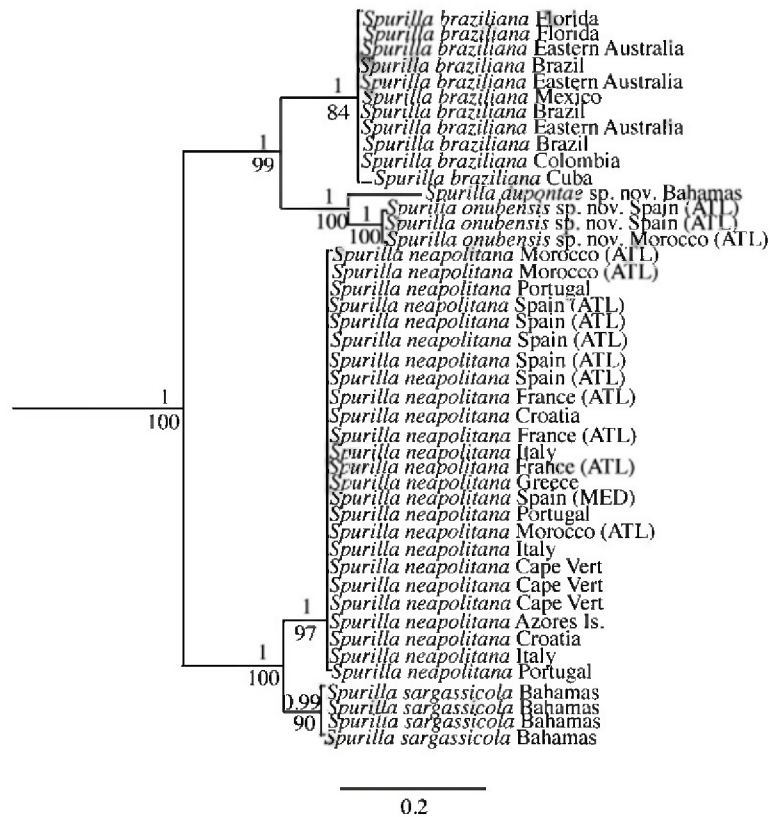
Externally, *S. dupontae* sp. nov. is easily distinguishable from the remaining members of this genus by its conspicuous reticulate pattern covering the whole body, including the entire rhinophores. Although this pattern is found to some extent in all *Spurilla* species, in *S. dupontae* sp. nov. it is more evident and is visible on the rhinophores. All other species of *Spurilla* have similar morphology in the receptaculum seminis (Fig. 3A–D), whereas in *S. dupontae* sp. nov. it is folded and convoluted (Fig. 3E).

MOLECULAR RESULTS

*Phylogenetics analyses*

The combined data set yielded a sequence alignment of 1448 positions. The ILD test showed no significant conflicting signal between the three genes ( $P = 0.001$ ). No saturation was observed across genes and codon positions (not shown). The combined tree provided better resolution than *H3*, *COI*, or *16S* separately (not shown). Although bootstrap values were lower than posterior probabilities in more inclusive clades, the topologies of the ML trees were congruent with the results yielded by Bayesian analyses, and thus ML trees are not shown. Figure 7 shows the phylogenetic hypothesis for *Spurilla* based on the combined data set represented by BI. The complete tree is given in Figure S1.





**Figure 7.** Phylogenetic hypothesis for the genus *Spurilla* based on the combined data set (*H3* + *COI* + *16S*), inferred by Bayesian inference (BI) analysis. Numbers above branches represent posterior probabilities from BI. Numbers below branches represent bootstrap values from maximum-likelihood (ML) analysis. Abbreviations: ATL, Atlantic Ocean; MED, Mediterranean.

In this study, Aeolidiidae was monophyletic (PP = 1; BS = 72), but the relationships among genera were not fully resolved. Excluding *Cerberilla*, all the genera were monophyletic and had maximum support (Figure S1).

*Spurilla* specimens all clustered together in a monophyletic group, with maximum support (PP = 1; ML = 100). This clade was divided into two subclades. Firstly, *S. braziliana* (PP = 1; ML = 84) was sister species of the group formed by *S. onubensis* sp. nov. (PP = 1; ML = 100) and *S. dupontae* sp. nov. (minimum uncorrected p-distance = 7.9% for *COI* among the three species). The second subclade was constituted by *S. neapolitana* (PP = 1; ML = 97) and *S. sargassicola* as its sister species (PP = 0.99; ML = 90; minimum uncorrected p-distance = 6.4% for *COI* between both species).

#### Population genetics analyses

For the AMOVA analysis of *COI* haplotypes, among-population differentiation explained 85.99% of the covariance component, whereas within-population differentiation explained 14.01% ( $\Phi_{ST} = 0.85987$ ,  $P =$

0.0000, based on 16 000 permutations). The pairwise  $F_{ST}$  values are summarized in Table 1.

## DISCUSSION

The main conclusion of the present study is that *Spurilla* is a monophyletic group, constituted so far by five different species. These species can be distinguished by a combination of morphological and molecular synapomorphies. Further support for species delimitations was obtained from the AMOVA and  $F_{ST}$  analyses. The AMOVA test showed significant genetic differentiation between the five species recognized here. The  $F_{ST}$  analysis shows significant differentiation between most species pairs, with the exception of the Pacific and Atlantic populations of *S. braziliana* (which are the same species), and *S. sargassicola* and *S. dupontae* sp. nov. The small sample size for the latter pair probably accounts for the non-significant results.

As *S. neapolitana* was the first species described (Delle Chiaje, 1841), it has been reported from numer-

**Table 1.** Results of the  $F_{ST}$  pairwise comparison of genetic differentiation among species with 16 000 permutations (lower triangular) and significance  $P$  values (upper triangular)

	<i>S. neapolitana</i>	<i>S. sargassicola</i>	<i>S. braziliiana</i> Atlantic	<i>S. braziliiana</i> Pacific	<i>S. dupontae</i> sp. nov.
<i>Spurilla neapolitana</i>	–	0 ± 0.0001	0 ± 0.0001	0 ± 0.0001	0 ± 0.0001
<i>Spurilla sargassicola</i>	0.79472	–	0.0003 ± 0.0001	0.00394 ± 0.0005	<b>0.31882 ± 0.0037</b>
<i>Spurilla braziliiana</i> Atlantic	0.92685	0.71037	–	<b>0.65881 ± 0.004</b>	0.00062 ± 0.0002
<i>Spurilla braziliiana</i> Pacific	0.93378	0.62231	–0.04386	–	0.00506 ± 0.0006
<i>Spurilla dupontae</i> sp. nov.	0.88004	0.28848	0.90186	0.91043	–

Significant  $P$  values after Bonferroni correction,  $P < 0.005$ . Non-significant values are set in bold.

**Figure 8.** Map of the range of the different species of *Spurilla*, including the localities where the specimens examined were collected.

ous locations. Specimens collected from areas far from the type locality were considered to belong to *S. neapolitana* mainly because of its accepted variability in colour pattern. Furthermore, other species of *Spurilla*, such as *S. braziliiana* or *S. sargassicola*, were considered to be within the chromatic variability of *S. neapolitana*, and were therefore regarded as junior synonyms. Our molecular data based on three molecular markers show the presence of five distinct and often allopatric species in the *S. neapolitana* species complex. These results are in agreement with Carmona *et al.* (2013), and highlight the need to carefully re-examine other species with broad geographic and chromatic ranges. This is the new paradigm of heterobranch taxonomy, species with broad and/or disjunct ranges constitute complexes of genetically distinct species. Other examples such as *Bulla striata*

Bruguère, 1792, *Chelidonura* spp., and *Navanax enigmaticus* (Bergh, 1893) were recently determined to be species complexes using molecular approaches (Malaquias & Reid, 2008, 2009; Ornelas-Gatdula *et al.*, 2011, 2012, respectively).

The new taxonomy of *Spurilla* presented here shows an intriguing combination of widespread species and endemics with restricted ranges (Fig. 8). Two species, *S. neapolitana* and *S. braziliiana* have broad ranges in the eastern and western Atlantic. The range of *S. neapolitana* is continuous, whereas *S. braziliiana* has a disjunct range, with populations in the eastern Pacific, the Hawaiian Islands, Japan, China, and Australia. Carmona *et al.* (2013) pointed out that the Pacific distribution of *S. braziliiana* could be the result of a recent human-mediated introduction, as it was first reported in the Pacific relatively

recently (Gosliner, 1980), and then a few years later in China, Japan, Australia, and Peru (Lin, 1992; Hamatani, 2000; Willan, 2006; Uribe & Pacheco, 2012, respectively). Additionally, the limited genetic and chromatic diversity found in Pacific specimens reinforces the hypothesis of a recent introduction. Although the chronology of these reports may indicate a westward spread, to test this hypothesis would require the study of haplotype diversity of Pacific and western Atlantic populations, which is beyond the scope of this study.

The phylogenetic analyses conducted herein provide important information to understand the evolutionary history of the species in the context of their geographic distribution. For example, *S. sargassicola* is found exclusively on floating sargasso in the western Atlantic (Gulf of Mexico and Bahamas), and is sister to the eastern Atlantic species *S. neapolitana*. Because *S. sargassicola* is the only pelagic species of Aeolidiidae, it is logical to assume that this is the result of the adaptation to pelagic sargasso habitat by this species, possibly leading to speciation. But because the ranges of *S. neapolitana* and *S. sargassicola* do not overlap, hypotheses of allopatric speciation cannot be rejected.

The two endemic species, *S. onubensis* sp. nov. and *S. dupontae* sp. nov., are sympatric in their entire range with the widespread *S. neapolitana* and *S. braziliana*, respectively. *Spurilla onubensis* sp. nov. and *S. dupontae* sp. nov. are sister to one another, and occur on opposite sides of the Atlantic Ocean, suggesting that these two species evolved allopatrically. *Spurilla dupontae* sp. nov. is apparently restricted to the Bahamas. Recent molecular studies on heterobranchs in the Bahamas have revealed the presence of several endemic species that are sister to widespread western Atlantic taxa (Ornelas-Gatdula *et al.*, 2011; Ornelas-Gatdula & Valdés, 2012). Bahamian endemics and their widespread sister taxa appear to occupy the same niche, suggesting that the speciation process was allopatric. Ornelas-Gatdula & Valdés (2012) discussed several possible scenarios leading to the evolution of Bahamian endemics, including a lack of connectivity between the Bahamas and the Caribbean, post-recruitment ecological factors, and historic vicariance. On the contrary, *S. dupontae* sp. nov. is sister to an eastern Atlantic species, suggesting that most likely there is no universal explanation for the origin of the endemic heterobranch fauna of the Bahamas.

Our phylogenetic analyses also show that species with similar external coloration are not necessarily closely related. For example, the two most similar *Spurilla* species from a chromatic point of view, *S. neapolitana* and *S. braziliana*, are not sister

species, suggesting that the colour pattern in these two taxa evolved independently.

Finally, regarding the morphological characters of *Spurilla* and the diagnosis of the genus based on the new molecular evidence, it appears *Spurilla* is well defined by the presence of lamellate rhinophores, cerata in arches usually curved inwards, and usually the presence of branches of the digestive gland forming a reticulate pattern. However, excluding the ornamentation of the rhinophores, the remaining characters are also present in other Aeolidiidae genera, such as *Berghia* (cerata in arches) and *Baeolidia* (cerata curved inwards in arches and reticulate pattern), which mean that these traits have evolved in different pathways within Aeolidiidae or were lost several times independently. Hence, perfoliate rhinophores is the only morphological synapomorphy found so far in *Spurilla*.

Combining morphological and molecular analyses, we conducted the most comprehensive review of *Spurilla* to date. This new understanding of the *S. neapolitana* species complex is vital for determining the evolutionary and ecological dynamics of widely distributed, closely related marine invertebrate species, and lays the framework for further studies in this field.

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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:

**Figure S1.** Phylogenetic hypothesis based on the combined data set (*H3* + *COI* + *16S*), inferred by Bayesian analysis.

**Table S1.** List of specimens used for phylogenetic analyses.