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Redescription of *Felimida elegantula* (Philippi, 1844) and a preliminary phylogeny of the European species of *Felimida* (Chromodorididae)

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ABSTRACT

Felimida elegantula (Philippi, 1844) was originally described as *Doris elegantula* based on the external morphology of a single specimen from Sicily (Italy). Since Philippi's description, this species has been recorded only a few times, always from the Mediterranean Sea, and without any detailed description of the internal morphology. According to a recent reassessment of the family Chromodorididae, all the eastern Pacific, Atlantic and Mediterranean species previously attributed to the genus *Chromodoris* Alder & Hancock, 1855 should be reallocated to *Felimida* Marcus, 1971. Here we present a morphological redescription of *F. elegantula* based on five specimens from Sardinia (Italy) as well as a molecular phylogeny using two mitochondrial (cytochrome *c* oxidase subunit I) and16S rRNA) and one nuclear (histone-3) marker. We aim to investigate phylogenetic relationships within '*Felimida*' from the Atlantic coast of Europe and the Mediterranean Sea.

INTRODUCTION

Doris elegantula Philippi, 1844 is a poorly known species and its generic placement and phylogenetic relationships have been controversial. It was originally described based on a single specimen from Sicily (Italy) and Philippi (1844) provided information only on its external morphology. In 1880, von Ihering identified one specimen, also from Italy, as *D. elegantula*, but Pruvot-Fol (1932) stated that this was a misidentification of *Diaphorodoris luteocincta* (Sars, 1870). In addition, Pruvot-Fol (1932) described the external morphology of a single specimen of *D. elegantula* from Villefranche-sur-Mer (France) but assigned it to the genus *Glossodoris* Ehrenberg, 1831. Although she described the buccal armature and radula of the specimen, no illustrations were supplied. Sordi (1970) collected one specimen of *G. elegantula* from the Ligurian Sea (Italy) and briefly described its buccal armature, radula, penis and the egg mass. Cattaneo-Vietti & Barletta (1984) transferred the species to *Chromodoris* Alder & Hancock, 1855 and later Cattaneo-Vietti, Chemello & Giannuzzi-Savelli (1990: pl. 3, fig. 1) gave a colour photograph of the specimen studied by Sordi (1970). Perrone (1993) found several specimens in Malta and, based on the occurrence of two distinct colour forms, proposed two subspecies: *C. elegantula elegantula*, with small dorsal red spots, and *C. elegantula polychroma*, with large dorsal spots. In addition, Perrone (1993) provided details and drawings of the radula, buccal armature and reproductive system. Later, Trainito (2003, 2005) published photographs of specimens from southern Sardinia (Italy). The most recent publication on *C. elegantula* is the record of one specimen from Turkey (Türkmen & Demirsoy, 2009).

A recent reassessment of the family Chromodorididae, based on molecular data, led Johnson & Gosliner (2012) to propose a new classification, with 14 valid genera. Among these, *Felimida* Ev. Marcus 1971 and *Dorisprismatica* d'Orbigny, 1839 were provisionally re-erected. *Felimida* was originally defined on the basis of the denticulation of the radular teeth and was said to be the only chromodorid genus bearing denticles on the inner side of the 1st to 4th innermost lateral teeth (Marcus, 1971: fig. 3).

However, Bertsch (1977) synonymized *Felimida* with *Chromodoris*, considering the differences in radular denticulation as a variation within *Chromodoris*. Johnson & Gosliner (2012), in a phylogenetic study based on two mitochondrial markers, proposed the use of the name *Felimida* for the eastern Pacific, Atlantic and Mediterranean species previously included in the genera *Chromodoris* and *Glossodoris*. However, the sampling of Atlantic chromodorids in that study was quite incomplete.

In the present contribution we focus on *Felimida elegantula*, providing a morphological redescription of the species. To test its generic placement and assess its phylogenetic relationships we have used two mitochondrial genes, cytochrome *c* oxidase subunit I (COI) and16S rRNA (16S), and one nuclear gene, histone-3 (H3), and have analysed the species with other externally similar species allocated to *Felimida* from the Atlantic coast of Europe and the Mediterranean Sea, and the type species of the genus from the eastern Pacific Ocean. We aim to improve understanding of the relationships within *Felimida* (*sensu* Johnson & Gosliner, 2012), with the inclusion of more species, more sequences and a nuclear marker (H3).

MATERIAL AND METHODS

Morphological studies

Six specimens of *Felimida elegantula* were obtained through scuba diving in Porto San Paolo, northeastern Sardinia, Italy. One specimen was preserved in 4% formalin for histology. The remaining specimens were preserved in 96% ethanol for morphological and molecular studies. Specimens were dissected by dorsal incision. Their internal features were examined using a dissecting microscope and drawn with the aid of a camera lucida. Special attention was paid to the morphology of the reproductive system. The buccal mass was removed and dissolved in 10% sodium hydroxide until the radula and the labial cuticle were isolated from the surrounding tissue. The radula and the labial cuticle were then rinsed in water, dried and mounted for examination under a Quanta 200 scanning electron microscope.

Samples for molecular analysis

Molecular analysis included 59 specimens, representing five genera of Chromodorididae and ten *Felimida* species (Table 1). Members of six additional genera were included for comparative purposes. Voucher specimens are held at the Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN), the California Academy of Sciences, San Francisco, USA (CASIZ), Colección Nacional de Moluscos, Universidad Nacional Autónoma de México, Mexico City, México (CNMO), Museo de Zoología de la Universidad de Costa Rica, San José, Costa Rica (MZUCR), Museo de Historia Natural de El Salvador, San Salvador, El Salvador (MHNES), the Natural History Museum of Crete, Crete, Greece (NHMC), the Zoologische Staatssammlung München (ZSM), and the Zoological Museum of Bergen, Bergen, Norway (ZMBN). We obtained 34 new sequences for both COI and for H3, and 35 for 16S. Sixty-seven additional sequences from 32 specimens were obtained from GenBank (27 for COI, 30 for 16S, and 10 for H3), with emphasis on specimens and sequences used by Johnson & Gosliner (2012). *Tritonia challengeriana* Bergh, 1884 was chosen as outgroup. The classification used in this study is based on Johnson & Gosliner (2012) and Carmona *et al.* (2013) (Table 1).

DNA extraction, amplification and sequencing

DNA extractions and PCR amplifications were performed at the Universidad de Cádiz (UCA), Spain and in the Zoologische Staatssammlung München (ZSM), Germany. DNA was extracted from foot tissue of specimens preserved with 70–100% ethanol, and performed using the DNeasy Blood and Tissue Kit Qiagen at UCA and NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co.) at ZSM, following the manufacturer's instructions. Partial sequences of COI, 16S and H3 were amplified by polymerase chain reaction (PCR) using LCO1490 and HCO2198 universal primers for COI (Folmer *et al.*, 1994), 16S ar-L and 16S br-H for 16S (Palumbi *et al.*, 1991) and H3AD5′3′ and H3BD5′3′ for H3 (Colgan *et al.*, 1998). The master mix for the PCR was prepared in the following order: nuclease-free water up to 25 µl volume reaction, 2.5 µl of Qiagen buffer, 2.5 µl of dNTP (2 mM), 5 µl of 'Q-solution' (Qiagen), 1.5–3.5 µM magnesium chloride, 1 µl of each forward and reverse primer (10 µM), 0.25 µl of DNA polymerase (250 units) and 2–3 µl of DNA. COI amplification was performed with an initial denaturation for 3 min at 94–95 °C, followed by 39–40 cycles of 30–45 s at 94 °C, 30–45 s at 46 °C (annealing temperature) and 1–2 min at 72 °C with a final extension of 5 min at 72 °C. 16S amplification was performed with an initial denaturation for 3 min at 94–95 °C, followed by 39 cycles of 39–45 s at 94 °C, 30–50 s at 45–51.5 °C (annealing temperature), 2 min at 72 °C, with a final extension of 5–10 min at 72 °C. H3 amplification was performed with an initial denaturation for 3 min at 95 °C, followed by 40 cycles of 45–60 s at 94–95 °C, 45 s at 50 °C (annealing temperature), 2 min at 72 °C, with a final extension of 10 min at 72 °C.

Successful PCR products obtained at UCA were purified and sequenced by Macrogen, Inc. PCR products obtained at ZSM were purified using the NucleoSpin Extract II (Macherey-Nagel GmbH & Co). Cycle sequencing using Big Dye 3.1 and the PCR primers (10 pm/µl) was conducted by the Genomic Service Unit of the Department of Biology, Ludwig-Maximilians-University Munich. All new sequences obtained were deposited in GenBank.

Molecular analyses

DNA sequences were assembled and edited using Geneious v. 6.1.6 (Drummond *et al.*, 2009). All the sequences were checked for contamination with BLAST (Altschul *et al.*, 1990) implemented in the GenBank database. To align the sequences we used MAFFT (Katoh, Asimenos & Toh, 2009). The alignments were checked by eye using MacClade v. 4.06 (Maddison & Maddison, 2005). Protein-coding sequences were translated into amino acids for confirmation of alignment. Pairwise uncorrected p-distance values between each taxon were calculated for the COI gene using PAUP v. 4.ob10 (Swofford, 2002). Uncorrected p-distances between all taxa, and level of saturation for first, second and third codon positions (p-distances against transitions plus transversions) were calculated in MEGA v. 5.0 (Tamura *et al.*, 2011) for the COI and H3 genes.

The most variable regions from the 16S rRNA alignment were removed in the first analyses, using both the default settings and the standard options for stringent and less stringent selection in Gblocks (Talavera & Castresana, 2007). When these regions were excluded from the analyses, the combined phylogenetic tree was poorly resolved with low nodal support. Therefore, final analyses were performed including all bases. Individual gene analyses and a concatenated analysis were performed. The best-fit models of evolution for each gene were determined using the Akaike information criterion (Akaike, 1974) implemented in MrModeltest v. 2.3 (Nylander, 2004). The GTR+I+G model was selected for the concatenated analysis.

Maximum likelihood (ML) analyses were performed using the software RAxML v. 7.0.4 (Stamatakis, 2006) and nodal support was assessed with nonparametric bootstrapping (BS) with 5000 replicates, random starting trees and parameters estimated from each dataset under the model selected for the original dataset. Bayesian inference analyses (BI) were conducted using MrBayes v. 3.1.2b (Ronquist & Huelsenbeck, 2003) for five million

Table 1. Specimens used in this study, with localities, museum voucher numbers and GenBank accession numbers (including the original museum voucher number, if available).

Species	Locality	Museum Voucher Number	COI	16S	H3
Tritonia challengeriana	Bouvet Island, Norway (EA)	CASIZ 171177 (GB)	HM162718.1	HM162643.1	HM162550.1
Piseinotecus gaditanus	Spain (EA)	MNCN 15.05/53704 (GB)	HQ616759	HQ616722	HQ616788
Spurilla neapolitana	Balearic Island, Spain (MED)	MNCN/ADN: 51961 (GB)	JX087582	JX087517	JX087655
Spurilla neapolitana	France (EA)	MNCN/ADN: 51969 (GB)	JX087574	JX087514	JX087650
Berghia verrucicornis	Morocco (EA)	MNCN 15.05/53686 (GB)	HQ616749	HQ616712	HQ616778
Berghia verrucicornis	Spain (EA)	MNCN 15.05/53687 (GB)	HQ616750	HQ616713	HQ616779
Triopha maculata	Marin County, Duxbury Reef, California, USA (EPAC)	CASIZ 181556 (GB)	HM162691.1	HM162601.1	HM162507.1
Triopha catalinae	San Francisco Yacht Harbor, San Francisco, USA (EPAC)	CASIZ 170648 (GB)	HM162690.1	HM162600.1	HM162506.1
Peltodoris nobilis	Pillar Point, San Mateo County, USA (EPAC)	CASIZ 182223 (GB)	EU982761	EU982816	HM162499.1
Noumea haliclona	Port Philip Bay, Australia (WPAC)	SAM D19269 (GB)	EF535117.1	EF534045.2	–
Felimare villafranca	Taghazout, Morocco (EA)	MNCN 15.05/70681	KJ911288	KJ911268	KJ911248
Felimare villafranca	Taghazout, Morocco (EA)	MNCN 15.05/70682	KJ911289	KJ911269	KJ911249
Felimare villafranca	Menorca, Cap Cavalleria, Spain (MED)	MNCN 15.05/70683	KJ911290	KJ911270	KJ911250
Felimare villafranca	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70684	KJ911291	KJ911271	KJ911251
Felimare villafranca	San García, Cadiz, Spain (Strait of Gibraltar)	MNCN 15.05/70695	KJ911292	KJ911272	KJ911252
Felimare villafranca	Ilhas do Martinhal, Algarve, Portugal (EA)	CASIZ 185127 (GB)	–	JQ727793.1	–
Felimare villafranca	Cadiz, Spain (EA)	GB	AJ223266.1	AJ225190.1	–
Felimare villafranca	Spain (EA)	GB	–	AF249237.1	–
Goniobranchus splendidus	Mooloolaba, Queensland, Australia (WPAC)	CASIZ 146039 (GB)	EU982738.1	EU982789.1	–
Goniobranchus splendidus	Mooloolaba, Queensland, Australia (WPAC)	SAM D19292 (GB)	EF535115.1	AY458815.1	–
Chromodoris strigata	Nosi Kalakjoro, Iles de Radama, Madagascar (IO)	CASIZ 175558 (GB)	JQ727857.1	JQ727739.1	–
Chromodoris strigata	Maricaban Island, Batangas, Philippines (WPAC)	CASIZ 158260 (GB)	JQ727856.1	JQ727738.1	–
Chromodoris aspersa	Mooloolaba, Queensland, Australia (WPAC)	SAM D19282 (GB)	–	AY458813.2	–
Chromodoris aspersa	Napili Bay, Maui, Hawaii (WPAC)	CASIZ 174975 (GB)	–	JQ727705.1	–
Chromodoris magnifica	Whitsundays, Queenslad, Australia (WPAC)	SAM D19290 (GB)	EF535110.1	EF534042.2	–
Chromodoris magnifica	Maricaban Island, Batangas, Philippines (WPAC)	CASIZ 157027 (GB)	EU982736.1	EU982787.1	–
Chromodoris magnifica	Mooloolaba, Queensland, Australia (WPAC)	CASIZ 144119 (GB)	JQ727852.1	JQ727731.1	–
Felimida edmundsi	Pedra Adalio, Príncipe Island, São Tomé and Príncipe (EA)	CASIZ 179385 (GB)	HM162686.1	HM162595.1	HM162501.1
Felimida edmundsi	Ilhéu Mosteiros, São Tomé and Príncipe (EA)	CASIZ 179394	KJ812351	KJ804240	KJ812364
Felimida edmundsi	Ilhéu Mosteiros, São Tomé and Príncipe (EA)	CASIZ 179411	KJ812352	KJ804241	KJ812365
Felimida edmundsi	Ilhéu Cabra, São Tomé (EA)	GB	EF535133.1	EF534061.2	
Felimida edmundsi	Azores, Portugal (EA)	ZMBN 81682	KJ812350	KJ804239	KJ812363
Felimida edmundsi	Azores, Portugal (EA)	ZMBN 81703	KJ812353	KJ804242	KJ812366
Felimida dalli	Santa Lucía Bay, Guerrero, Mexico (EPAC)	CNMO 4964	KJ911293	KJ911267	KJ911247
Felimida dalli	Guanacaste, Punta Carbon, Costa Rica (EPAC)	CASIZ 175428 (GB)	EU982741.1	EU982793.1	–
Felimida dalli	Tres Hermanas Island, Costa Rica (EPAC)	CASIZ 175439 (GB)	JQ727869.1	JQ727751.1	–
Felimida purpurea	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70693	KJ911285	–	KJ911244
Felimida purpurea	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70694	KJ911286	KJ911265	KJ911245
Felimida purpurea	Ilhéu dos Mosteiros, São Miguel Island, Azores, Portugal (EA)	ZMBN 87934	KJ812354	KJ804243	KJ812367
Felimida purpurea	Cadiz, Spain (EA)	GB	AJ223260.1	AJ225184.1	
Felimida krohni	Del Rey Island, Chafarinas, Spain (MED)	MNCN 15.05/70689	KJ911274	KJ911254	KJ911233
Felimida krohni	Congreso Island, Chafarinas, Spain (MED)	MNCN 15.05/70690	KJ911275	KJ911255	KJ911234
Felimida krohni	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70691	KJ911276	KJ911256	KJ911235
Felimida krohni	Guetaria Bay, Basque Country, Spain (EA)	MNCN 15.05/70697	KJ911277	KJ911257	KJ911237
Felimida krohni	Italy (MED)	MNCN 15.05/70698	KJ911278	KJ911258	KJ911236
Felimida krohni	Murcia, Spain (MED)	GB	AY345036.1	–	–
Felimida krohni	Spain (EA)	GB	AF249805.1	AF249239.1	–
Felimida luteorosea	Del Rey Island, Chafarinas, Spain (MED)	MNCN 15.05/70692	KJ911283	KJ911263	KJ911242
Felimida luteorosea	Guetaria Bay, Basque Country, Spain (EA)	MNCN 15.05/70696	KJ911284	KJ911264	KJ911243
Felimida luteorosea	Spain (MED)	GB	AF249815.1	–	–
Felimida luteorosea	Greece (MED)	NHMC 52.116	KJ812355	KJ804244	–
Felimida luteopunctata	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70685	KJ911279	KJ911259	KJ911238
Felimida luteopunctata	Santa Maria Beach, Cadiz, Spain (EA)	MNCN 15.05/70686	KJ911280	KJ911260	KJ911239
Felimida luteopunctata	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70687	KJ911281	KJ911261	KJ911240

Continued

Table 1. *Continued*

Species	Locality	Museum Voucher Number	COI	16S	H3
Felimida luteopunctata	Santa María Beach, Cadiz, Spain (EA)	MNCN 15.05/70688	KJ911282	KJ911262	KJ911241
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	MNCN 15.05/60113N	KJ812356	KJ804245	KJ812368
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	MNCN 15.05/60113	–	KJ804246	KJ812369
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	MNCN 15.05/69821	KJ812357	KJ804247	KJ812370
Felimida elegantula	Porto San Paolo, Sardinia, Italy (MED)	ZSM Mol 20130570	KJ812358	KJ804248	KJ812371
Felimida sphoni	La Unión Beach, Gulf of Fonseca, El Salvador (EPAC)	MHNES 90-0425	–	KJ804249	KJ812372
Felimida sphoni	Santa Lucía Bay, Guerrero, Mexico (EPAC)	CNMO 4965	KJ911287	KJ911266	KJ911246
Felimida sphoni	Herradura Beach, Punteras, Costa Rica (EPAC)	MZUCR8099	KJ812359	KJ804250	KJ812373
Felimida sphoni	Guanacaste, Punta Carbón, Costa Rica (EPAC)	CASIZ 175431 (GB)	–	JQ727736.1	–
Felimida baumanni	Guanacaste, Costa Rica (EPAC)	CASIZ 175434	KJ812360	KJ804251	KJ812374
Felimida baumanni	Reserva Natural Absoluta Cabo Blanco, Punteras, Costa Rica (EPAC)	MZUCR9023	KJ812361	KJ804252	–
Felimida baumanni	Tamarindo Beach, Guanacaste, Costa Rica (EPAC)	CASIZ 175433 (GB)	JQ727866.1	JQ727748.1	–
Felimida britoi	Ilhéu dos Mosteiros, São Miguel Island, Azores (EA)	ZMBN 87950	KJ812362	KJ804253	KJ812375
Felimida britoi	Madeira, Portugal (EA)	ZSM Mol 20130740	KJ911273	KJ911253	KJ911232

Abbreviations: EA, eastern Atlantic Ocean; EPAC, eastern Pacific Ocean; GB, GenBank; IO, Indian Ocean; MED, Mediterranean; WPAC, western Pacific Ocean. Asterisks indicate newly generated sequences.

generations with two independent runs and sampling frequency of 1000. The models implemented were those estimated with MrModeltest v. 2.3. The combined dataset was partitioned among genes and the 'unlink' command was used to allow all parameters to vary independently within each partition.

Convergence was diagnosed graphically by plotting for each run the likelihood against the number of generations using the software Tracer v. 1.4.1 (Drummond & Rambaut, 2007). For each analysis, the first 1250 trees were discarded as 'burn-in'. Nodal support was assessed with posterior probabilities (PP). Only nodes supported by BS $\geq$75 and PP $\geq$0.90 were considered as resolved.

Two species-delimitation analyses were made including *F. elegantula* and its closest related species *F. luteopunctata* (Gantès, 1962) and *F. luteorosea* (Rapp, 1827). The automatic barcode gap discovery (ABGD) method (Puillandre *et al*., 2012) was performed using the online version of the software (available at http://wwwabi.snv.jussieu.fr/public/abgd/) with the default settings to generate a preliminary partition of sequences, using the COI alignment. In addition the species-delimitation plugin (Masters, Fan & Ross, 2011) in Geneious was used to provide a statistical framework to assess putative species in the phylogenetic analyses, using the Bayesian concatenated tree without modifications, with special interest in the same species.

RESULTS

Molecular results

The combined dataset based on COI, H3 and 16S yielded a sequence alignment of 1477 positions. No saturation was observed across genes and codon positions, not even in the third codon position (not shown). The resulting combined tree provided better resolution than H3, COI or 16S separately (not shown). Figure 1 shows the phylogenetic hypothesis based on the combined dataset constructed by Bayesian Inference. The topology of the ML tree was identical (not shown). Chromodorididae are monophyletic with high support in both Bayesian and ML analyses (PP = 1, BS = 86), but the relationships between species and genera were not well resolved (Fig. 1). Within Chromodorididae there is a polytomy consisting of: *Noumea haliclona* (Burn, 1957); *Felimare villafranca* (Risso, 1818); *Goniobranchus splendidus* (Angas, 1864); a clade of *Chromodoris magnifica* (Quoy & Gaimard, 1832), *C. strigata* Rudman, 1982 and *C. aspersa* (Gould, 1852) (PP = 1, BS = 100); *Felimida baumanni* (Bertsch, 1970); a clade including *Felimida edmundsi* (Cervera, García-Gómez & Ortea, 1989) and *Felimida dalli* (Bergh, 1879) (PP = 1, BS = 85; a clade including *Felimida krohni* (Vérany, 1846) and *F. purpurea* (Risso in Guérin, 1831) (PP = 1, BS = 94); and a clade containing the remaining species of *Felimida* included in this study. Philippi's *Doris elegantula* nested in this last clade together with the type species of the genus, *Felimida sphoni* Marcus, 1971. *Felimida elegantula*, *F. luteopunctata* and *F. luteorosea* were retrieved in a common clade (PP = 1, BS = 100). Apart from differences in morphology and external colour pattern, the analysis using the species-delimitation plugin (Masters *et al*., 2011) in Geneious confirmed *F. elegantula*, *F. luteopunctata* and *F. luteorosea* as distinct species. For this clade, 'P ID (Liberal)' minimum values were $\leq$0.91, i.e. a more than 90% chance of correctly placing an unknown specimen in its *a priori* designated species. For *F. elegantula* and *F. luteopunctata* these values were higher, $\leq$0.97 and $\leq$0.98, respectively (Table 2). The ABGD analysis recovered nine partitions with three groups each: one for *F. elegantula*, one for *F. luteopunctata* and one for *F. luteorosea* specimens. The prior maximal distance (P) ranged between 0.001 and 0.03. All tree topologies (from concatenated and single-gene analyses) supported monophyly of *F. elegantula*. The minimum uncorrected p-distances for COI between the *Felimida* species was 5.01% (*F. elegantula* – *F. luteorosea*) and the maximum 21.58% (*F. sphoni* – *F. purpurea*) (Table 3). Distances between the outgroup species *T. challengeriana* and the species of Chromodorididae ranged from 19.69% (*F. edmundsi*) to 23.58% (*F. purpurea*) (data not shown).

SYSTEMATIC DESCRIPTION

Chromodorididae Bergh, 1891

Felimida Ev. Marcus 1971

Diagnosis: Unicuspidate lateral teeth, many denticles on outer side, denticles on the inner side of the lateral teeth 1–4 (Marcus, 1971).

Felimida elegantula (Philippi, 1844)

(Figs 2–4)

Doris elegantula Philippi, 1844: 80, pl. 19, fig. 8.
Glossodoris elegantula—Pruvot-Fol, 1932: 327.
Chromodoris elegantula—Cattaneo-Vietti & Barletta, 1984: 205.

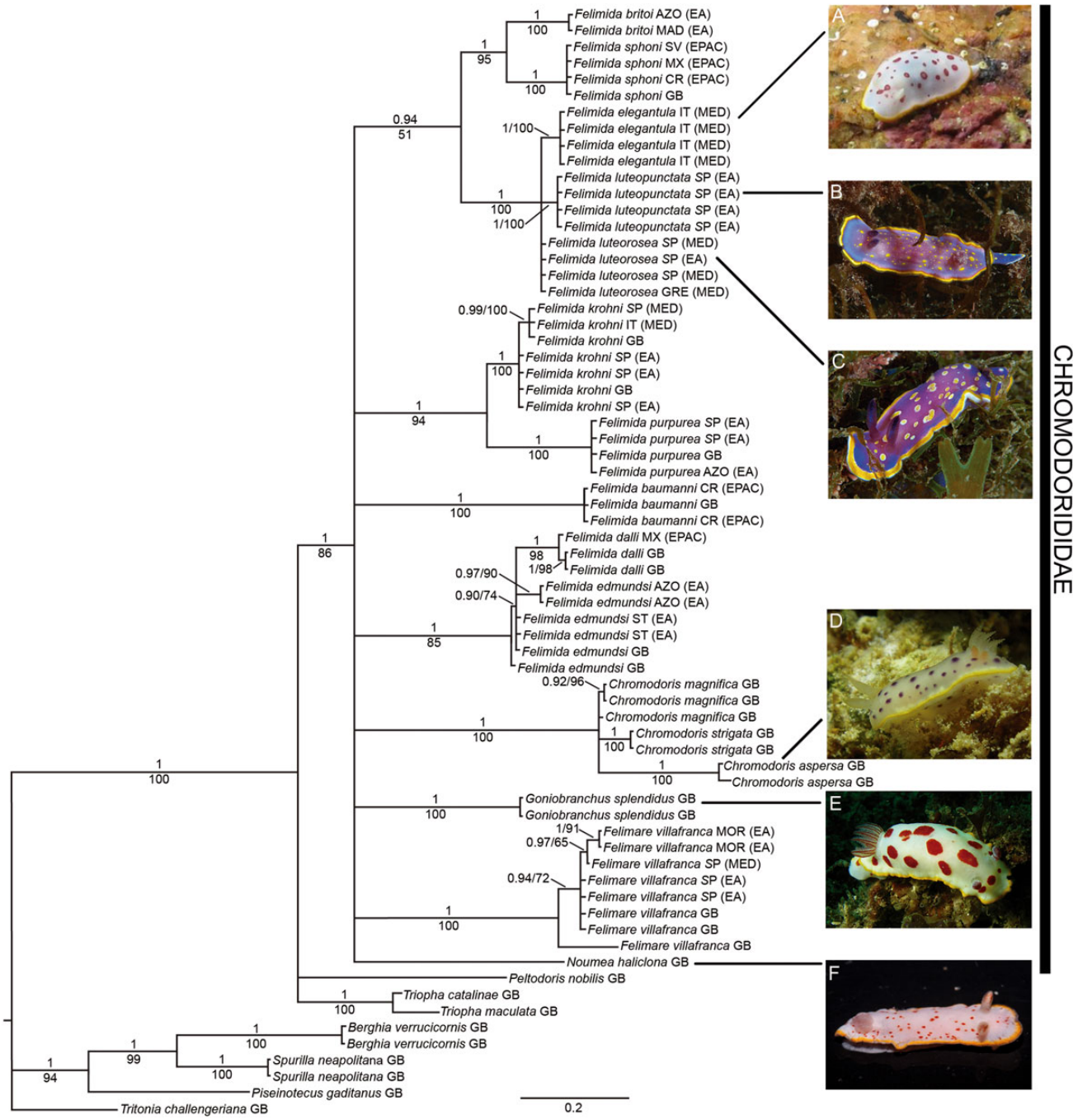

Figure 1. Phylogenetic hypothesis based on the combined dataset (H3+COI+16S) inferred by Bayesian analysis. Numbers above branches are posterior probabilities. Numbers below branches are bootstrap values. Abbreviations: ATL, Atlantic Ocean; AZO, Azores; CR; Costa Rica; GB, GenBank, GRE, Greece; IT, Italy; MAD, Madeira; MED, Mediterranean; MX, Mexico; MOR, Morocco; EA, Eastern Atlantic; EPAC, Eastern Pacific; SP, Spain; ST, São Tomé; SV, El Salvador. Photographs: **A.** *Felimida elegantula* (MNCN 15.05/60113; photo by E. Trainito). **B.** *F. luteopunctata* (photo by M. Martínez Chacón). **C.** *F. luteorosea* (photo by M. Martínez Chacón). **D.** *Chromodoris aspersa* (photo by S. Kahlbrock). **E.** *Goniobranchus splendidus* (photo by S. Kahlbrock). **F.** *Noumea haliclona* (photo by D. Aston).

Table 2. Species delimitations results for *Felimida* species, based on Bayesian analysis of concatenated sequences.

Species	Closest species	Monophyly	Intra Dist	Inter dist-closest	Intra/inter	P ID (strict)	P ID (liberal)
F. elegantula	*F. luteorosea*	Yes	0.003	0.060	0.06	0.83	0.97
F. luteopunctata	*F. luteorosea*	Yes	0.001	0.051	0.03	0.85	0.98
F. luteorosea	*F. luteopunctata*	Yes	0.014	0.051	0.28	0.68	0.91

Table 3. Minimum and maximum pairwise uncorrected p-distances for COI between chromodorid species.

Species	*Felimida britoi* (EA)	*Felimida sphoni* (EPAC)	*Felimida elegantula* (MED)	*Felimida luteopunctata* (EA)	*Felimida luteorosea* (EA, MED)	*Felimida krohni* (MED)	*Felimida purpurea* (EA)	*Felimida baumanni* (EPAC)	*Felimida dalli* (EPAC)	*Felimida edmundsi* (EA)	*Chromodoris magnifica* (WPAC)	*Chromodoris strigata* (WPAC)	*Goniobranchus splendidus* (WPAC)	*Felimare villafranca* (EA, MED)	*Noumea haliclona* (WPAC)
F. britoi	0														
F. sphoni	15.2–15.6	0													
F. elegantula	16.1–16.3	18.1–18.4	0												
F. luteopunctata	16.4–16.9	18.4–18.5	6.7–7.0	0											
F. luteorosea	15.1–16.9	17.5–18.4	5.0–6.0	5.3–5.8	0										
F. krohni	17.5–19.0	17.9–18.6	15.7–17.3	17.2–17.8	15.7–18.1	0									
F. purpurea	19.0–20.1	20.6–21.6	18.4–20.1	16.9–18.4	16.4–18.7	15.5–17.8	0								
F. baumanni	16.7–17.0	18.2–19.6	17.6–18.1	17.9–18.4	16.9–17.9	16.9–17.8	18.8–20.4	0							
F. dalli	18.4–19.1	18.8–20.2	16.3–17.3	17.6–17.8	16.1–17.3	15.2–17.3	17.5–19.4	16.0–16.6	0						
F. edmundsi	17.2–17.8	17.6–18.9	15.2–16.3	17.0–17.8	15.2–16.7	15.0–16.9	18.9–20.2	16.3–17.2	7.4–8.3	0					
C. magnifica	16.8–18.1	17.8–20.5	16.9–17.6	16.7–17.0	15.8–17.5	15.8–17.3	17.8–18.8	15.9–17.6	16.7–17.8	17.2–18.2	0				
C. strigata	17.6–18.1	19.0–20.2	17.8–18.1	17.0–17.3	16.9–17.5	17.9–18.8	19.3–19.9	18.1–18.4	17.3–17.5	17.9–18.8	6.8–7.7	0			
G. splendidus	17.1–18.1	16.4–18.4	14.6–15.6	15.2–16.0	15.8–16.7	17.0–18.1	16.0–17.0	17.8–18.2	14.9–15.8	14.8–15.4	16.4–17.1	16.1	0		
F. villafranca	17.6–19.7	18.7–19.6	17.5–17.9	17.0–18.2	16.9–18.1	17.4–19.4	18.0–19.6	19.6–20.8	15.4–17.8	15.2–16.9	16.9–19.4	16.8–18.7	17.6–19.6	0	
N. haliclona	15.9–16.1	16.8–17.4	16.3	17.3	16.6–17.5	16.0–17.0	17.2–18.4	18.2–18.4	16.1–16.7	15.4–15.8	19.7–21.0	20.7–20.8	16.3	16.3–17.5	0

Abbreviations: MED, Mediterranean; EA, Eastern Atlantic; WA, Western Atlantic; EPAC, Eastern Pacific; WPAC, Western Pacific.

Figure 2. Living specimen of *Felimida elegantula*. Porto San Paolo, northeastern Sardinia, Italy. (MNCN 15.05/60113; photo by E. Trainito).

Type material: Not located and believed lost. Thus, we designate here as neotype the specimen MNCN 15.05/60113N (preserved length 14 mm, dissected; 4–5 m depth, 10 Dec. 2011, Porto San Paolo, Sardinia, Italy, coll. E. Trainito).

Material examined: 5 specimens, Porto San Paolo, northeastern Sardinia, Italy: MNCN 15.05/60113N (10 Dec. 2011; preserved length 14 mm, dissected); MNCN 15.05/60113 (10 Dec. 2011; 9 mm); MNCN 15.05/60113 (10 Dec. 2011; 10 mm, dissected); ZSM Mol 20130570 (10 Oct. 2013; 5 mm); MNCN 15.05/69821 (10 Oct. 2013; 7 mm, dissected).

External morphology (Fig. 2): Body oval, elongate. Living animals white with several small red spots irregularly covering notum, lateral side of foot and tail; opaque white patches over notum; mantle edge yellow with thin opaque white band on inner side. Posterior end of foot not covered by notum. Mantle dermal formations (MDFs) not very conspicuous around mantle edge, absent at anterior region. Rhinophores with up to 13 lamellae. Gill with six pale white unipinnate branchial leaves, each with opaque white rachis. Completely retractile rhinophores and gills.

Internal anatomy (Fig. 3): Radular formulae of three specimens: 38 × 31.1.31 (MNCN 15.05/60113N, 14 mm), 34 × 20.1.20 (MNCN 15.05/69821, 7 mm); 29 × 27.1.27 (MNCN 15.05/60113, 10 mm). Rachidian teeth small but clearly visible, triangular, without denticulation (Fig. 3A). Innermost lateral teeth bifid; inner cusp broad; outer cusp slightly curved downwards with 5–7 denticles (Fig. 3A). Median lateral teeth with 5–8 denticles on inner face (Fig. 3B). Outermost lateral teeth with reduced broad base and 7–10 denticles at tip of each tooth (Fig. 3C). Labial cuticle brown, generally with bifid rodlets, but sometimes simple and bifid rodlets equally distributed (Fig. 3D).

Reproductive system (Fig. 4D) hermaphroditic, triaulic. Anterior portion occupying a relatively small space between buccal mass and digestive gland. Hermaphrodite duct flattened and short; ampulla moderately long, thin, centrally folded

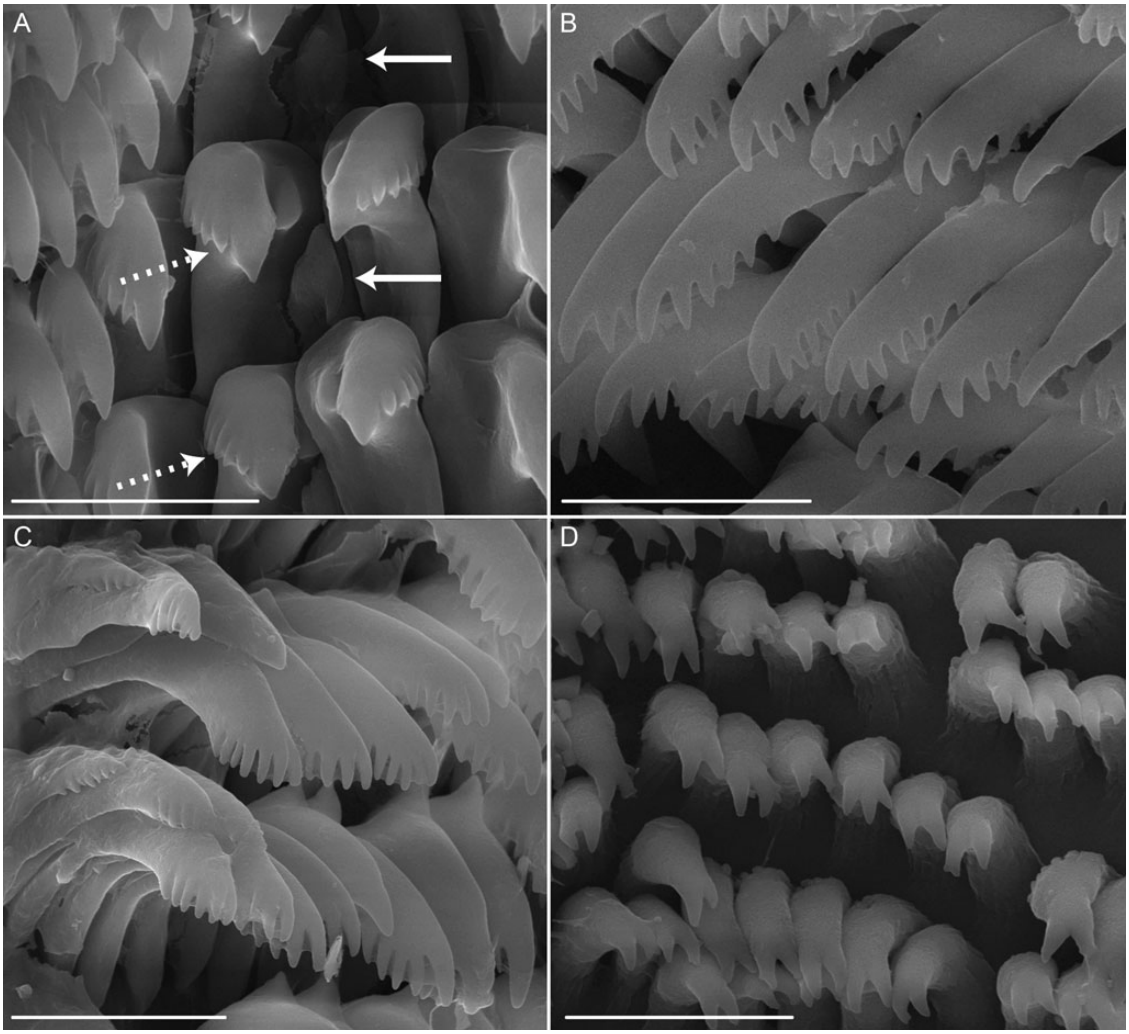

Figure 3. Scanning electron microscopes of *Felimida elegantula*. **A.** Detail of rachidian teeth (arrows) and denticles of the innermost lateral teeth (dashed arrows) (MNCN 15.05/69821). **B.** Median lateral teeth (MNCN 15.05/69821). **C.** Outermost lateral teeth (MNCN 15.05/69821). **D.** Elements of the armature of the labial cuticle (MNCN 15.05/69821). Scale bars: **A, B, C** = 25 μm; **D** = 12.5 μm.

(Fig. 4B, C). Prostate elongated, located ventrally to bursa copulatrix; deferent duct long, with many folds; transition between deferent duct and penial portion well demarcated (Fig. 4C); penial portion wide, located ventral to female gland and vagina, lacking any of accessories such as penial spines or glands. Vagina wide, moderately long (Fig. 4A, B); seminal receptacle small, pyriform (Fig. 4B). Bursa copulatrix very large, rounded (Fig. 4A). Uterine duct moderately long, large (Fig. 4B), inserting into female gland mass near to oviduct. Oviduct short (Fig. 4C). Female gland mass small, nidamental region with a rounded portion, ventral to vagina. Vestibular gland near orifice of female gland (Fig. 4A–C).

Geographical distribution (Fig. 5): *Felimida elegantula* has rarely been recorded since its original description (Philippi, 1844). It is only known from the Mediterranean Sea and has been recorded in Spain: Malgrats Islands (Balearic Islands) (Vives, 2007); France: Villafranche-sur-Mer (Pruvot-Fol, 1932); Italy: Palermo (Sicily) (Philippi, 1844, type locality), Porto San Paolo (present study), Cagliari and Tavolara (Sardinia) (Trainito, 2003; Piras, 2005), Secche della Meloria (Sordi, 1970), Pantelleria Island (Picchetti, 2000); Maltese archipelago (Cachia, Mifsud & Sammut, 1993; Perrone, 1993; Sammut & Perrone, 1998); and Turkey: Adrasan (Türkmen & Demirsoy, 2009).

DISCUSSION

Felimida elegantula was described by Philippi (1844) based on a single specimen from Sicily. The holotype was described as having a rectangular, opaque white body with dorsal small dark spots, yellow mantle edge, 11 branchial leaves and white rhinophores (Philippi, 1844). The colour pattern of our specimens from Porto San Paolo resembles Philippi's description, but also Perrone's (1993) *F. elegantula polychroma* regarding the size of the spots. According to this last author, the external differences between his two subspecies, *F. elegantula elegantula* and *F. elegantula polychroma*, were the larger spots and the presence of blotches in the latter. Since no specimens matching Perrone's description of *F. elegantula elegantula* are available for molecular analysis, it is not possible to test if his subspecies are intraspecific variants or different species.

Regarding the internal anatomy, Philippi (1844) did not present any data in the original description. Subsequently, Pruvot-Fol (1932, 1954), Sordi (1970) and Perrone (1993) reported some information about the number of radular teeth per row, but only Perrone (1993) gave a complete radular formula for *F. elegantula polychroma*, reporting 48 rows in one 27 mm specimen (48 × 52.0.52). None of these authors clearly stated the presence of a rachidian tooth, although Sordi (1970:

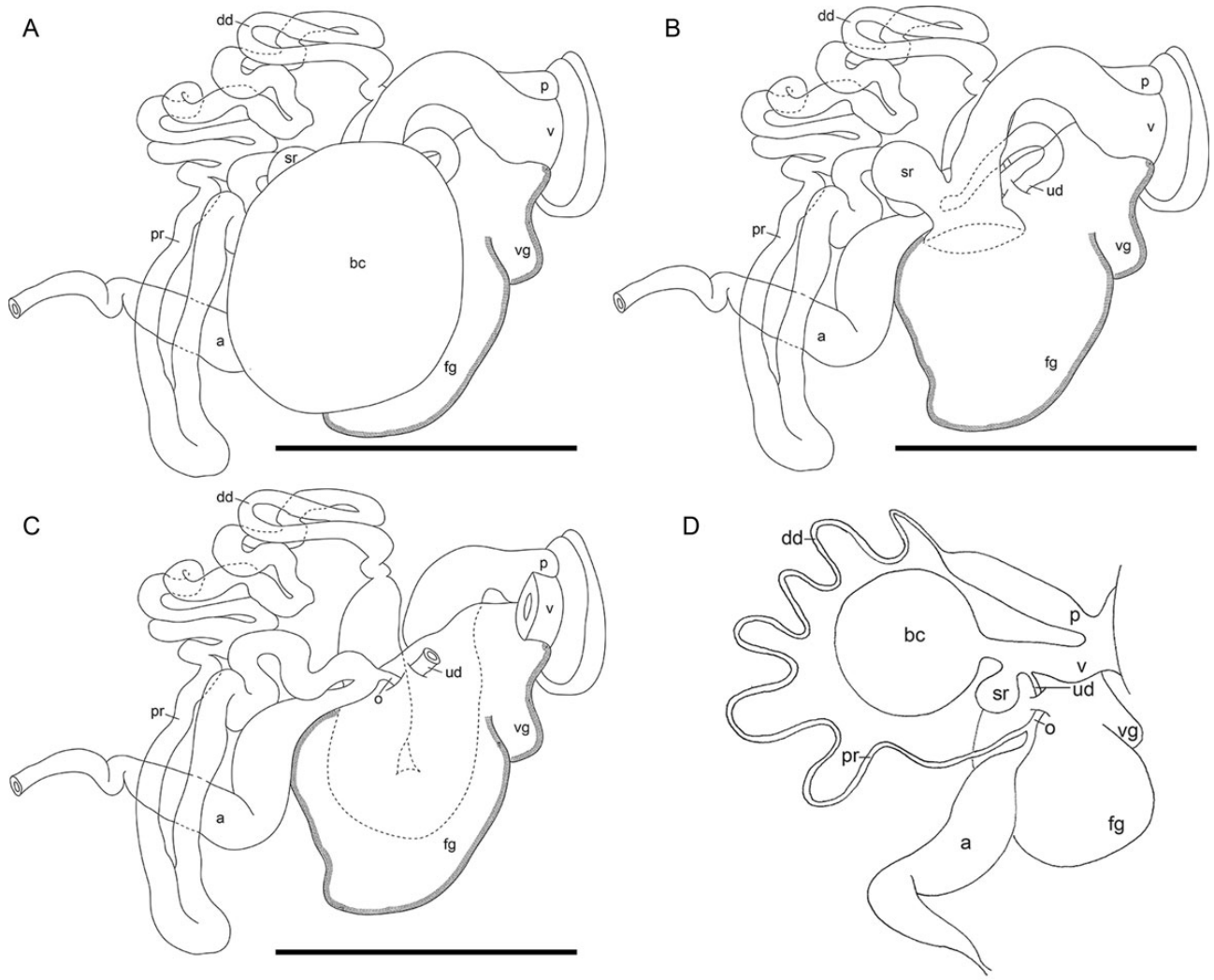

Figure 4. Reproductive system of *Felimida elegantula* (MNCN 15.05/60113N). **A.** Complete reproductive system. **B.** Partial view of reproductive system; bursa copulatrix removed. **C.** Partial view of reproductive system; bursa copulatrix, vagina, receptaculum seminis and uterine duct removed. **D.** Schematic drawing of complete reproductive system. Scale bars = 1.0 mm. Abbreviations: a, ampulla; bc, bursa copulatrix; dd, deferent duct; fg, female gland mass; o, oviduct; p, penis; pr, prostate; sr, receptaculum seminis; ud, uterine duct; v, vagina; vg, vestibular gland.

24) mentioned a "dente mediano quasi impercettibile", while giving the formula 50–0–50, and Perrone (1993: 75) stated "rachidiano è ridotto ad un semplice ispessimento, scarsamente visibile". Cattaneo-Vietti *et al.* (1990) first gave a radular formula including a rachidian tooth (38 × 35.1.35), which was described as subtriangular and evident. This description matches the rachidian teeth of our specimens. Concerning the armature of the labial cuticle, Perrone (1993) depicted the elements as curved and bifid, but in our specimens there are two different elements, some bifid and some with only one cusp. Perrone (1993) also stated that *F. elegantula polychroma* has the same kind of elements as *F. elegantula elegantula*.

The only information given by Sordi (1970) about the reproductive system was that the penis was unarmed. Perrone (1993) also mentioned that the penes of *F. elegantula elegantula* and *F. elegantula polychroma* were unarmed and that the female gland of the latter was easily separated from the bursa copulatrix and the deferent duct. This was also observed in the specimen studied in the present work (MNCN 15.05/60113N). Ours is the first study to give a complete and detailed description of the reproductive system of *F. elegantula*. The presence of a vestibular gland was not described by Perrone (1993), but it was observed in other species of the genus, e.g. in *F. luteopunctata* (Cervera, García-Gómez & Ortea, 1989), *F. luteorosea* (García-Gómez, 2002), and *Felimida corimbae* (Ortea, Gofás & Valdés, 1997).

Felimida elegantula clearly differs from other chromodorids in the Mediterranean Atlantic, by its unique and characteristic colour pattern and morphological features. The rachidian tooth is present in other Mediterranean species such as *F. britoi* (Ortea & Pérez, 1983), *F. luteopunctata* and *F. luteorosea*, while in *F. purpurea* it was observed by Cattaneo-Vietti *et al.* (1990), but not by García-Gómez (2002). The reproductive system of the species in the clade of *F. luteopunctata*, *F. luteorosea* and *F. elegantula* (Fig. 1) is quite similar, but with differences in the width and length of the vagina and in the shape of the receptaculum seminis. In *F. elegantula* the vagina is very wide and short, while in *F. luteopunctata* and *F. luteorosea* it is short but thin (Cervera *et al.*, 1989; García-Gómez, 2002). The vagina of *F. britoi* is also short and thin (Ortea & Pérez, 1983). *Felimida purpurea* and *F. krohni* have a thin and very elongated vagina (García-Gómez, 2002). In addition, the receptaculum seminis in *F. elegantula* is quite different from other Atlantic *Felimida* as it is almost rounded, while in others such as *F. luteopunctata*, *F. luteorosea*, *F. purpurea*, *F. britoi* and *F. krohni* it is elongated and sausage-shaped (Ortea & Pérez,

Figure 5. Distribution of *Felimida elegantula* according to published literature and photographs (see text).

1983; Cervera *et al*., 1989; García-Gómez, 2002). Two externally similar *Felimida* species from Angola, *F. corimbae* and *F. ocellata* (Ortea, Gofás & Valdés, 1997), also show differences from *F. elegantula* since both have a short but thin vagina and an elongated receptaculum seminis (very long in *F. corimbae*) (Ortea *et al*., 1997). The validity of *F. elegantula* as a distinct and valid species is supported by the monophyly of our newly collected specimens shown in the single and combined gene trees and by the species delimitation analyses.

In their molecular study, Johnson & Gosliner (2012) included nine putative *Felimida* species from the known distribution for this genus and, although these did not form a monophyletic clade based on mitochondrial COI and 16S markers, they nevertheless re-erected this genus. Our extended dataset includes 10 *Felimida* species with 32 additional specimens and additional sequences which include a nuclear gene for the first time (34 new sequences for COI, 35 for 16S gene, 34 for H3). Nevertheless, analyses of our combined dataset still did not recover *Felimida* as monophyletic, instead showing a polytomy with other chromodoridid groups, corroborating the previous ambiguous results. For now, we have decided to allocate *Doris elegantula* to *Felimida*, since the species clusters in the same clade as the type species, *F. sphoni*, from the eastern Pacific. A better resolution of the phylogeny of the eastern Pacific and Atlantic chromodoridids awaits comprehensive sampling of species from throughout these regions.

It is notable that in our study *F. elegantula* clustered together with the two other spotted *Felimida* species from the Atlantic (*F. luteopunctata* and *F. luteorosea*) (Fig. 1), suggesting that, regardless of the colour, the spotted pattern of these chromodoridids may have a common origin. The close relationship between *F. krohni*, *F. purpurea* and *F. luteorosea* shown by Johnson & Gosliner (2012) was not recovered in our study nor in that by Valdés *et al*. (2011). Interestingly, Rudman (1983) remarked on the external resemblance of *F. elegantula* to some Indo-Pacific species such as *Goniobranchus splendidus* (as *Chromodoris splendida*) and *Chromodoris aspersa* (Fig. 1), grouping them in his '*Chromodoris splendida* colour group'. Ortea *et al*. (1997) also noted the similarity in colour of *F. elegantula*, *F. ocellata* (as *Glossodoris ocellata*) and *F. corimbae* (as *Chromodoris corimbae*) with Indo-Pacific species of Rudman's *Chromodoris splendida* colour group. However, Rudman (1983) also commented that it would be unlikely that species with such wide geographic separation (Mediterranean and Indo-west Pacific) could be closely related. Our results corroborate Rudman's statement, with Indo-Pacific species clustering together and distant from Atlantic and Mediterranean ones.

The uncorrected p-distances for COI between members of *Felimida* ranged from 5.01% to 21.58%, some of them high if compared with typical interspecific values (mean 11.2 $\pm$ 5%) (Hebert, Ratnasingham & Waard, 2003), and with those found between other heterobranch species [7% between two '*Glossodoris*' species (Valdes *et al*., 2011); 10% for *Bulla* (Malaquias & Reid, 2008); 10–20% for sacoglossans (Krug, Händeler & Vendetti, 2011)].

In order to resolve these phylogenetic questions the dataset for *Felimida* (*sensu* Johnson & Gosliner, 2012) should be increased to include as many species as possible, in particular *F. macfarlandi* (Cockerell, 1902) from the eastern Pacific, *F. rolani* (Ortea, 1988) from the eastern Atlantic and *F. grahami* (Thompson, 1980) and *F. binza* (Ev. Marcus & Er. Marcus, 1963) from the tropical western Atlantic.

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