



A multilocus view on Mediterranean aeolid nudibranchs (Mollusca): Systematics and cryptic diversity of Flabellinidae and Piseinotecidae

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ABSTRACT

Recent molecular studies revealed high level of endemism and numerous cryptic species within opisthobranchs, with Mediterranean taxa clearly understudied. Here we used genetic data from both mitochondrial and nuclear gene fragments as well as morphological data from taxonomically relevant characters to investigate the phylogenetic relationships and systematics of Mediterranean taxa of the Flabellinidae and Piseinotecidae families. Phylogenetic analyses based on Bayesian and Maximum-Likelihood methods indicate that Flabellinidae and Piseinotecidae taxa and species within the genera *Flabellina*, *Calmella* and *Piseinotectus* do not form monophyletic clades. These results are supported by our morphological analyses which allowed the re-evaluation of the triseriate radula condition in Piseinotecidae and *Calmella* taxa and their inclusion in the genus *Flabellina* as *Flabellina gaditana* **comb. nov.** (synonym of *F. confusa*), *Flabellina gabinierei* **comb. nov.** and *Flabellina cavolini* **comb. nov.** Species delimitation and barcoding gap analyses allowed uncovering cryptic species within *Flabellina gracilis* (Alder and Hancock, 1844), *F. trophina* (Bergh, 1890), *F. verrucosa* (M. Sars, 1829) and *F. ischitana* Hirano and Thompson, 1990, the latter with an Atlantic form which is under description. This study corroborates the relevance of combining molecular and morphological data from multiple populations and species in the assessment of nudibranch diversity and classification.

1. Introduction

Biodiversity of the Mediterranean Sea is likely underestimated. As already pointed out for other marine ecosystems, recent investigations suggest that Mediterranean marine biodiversity may be much higher than earlier estimated, since the phenomenon of cryptic species represents an important hidden source of diversity in marine systems (Lee and Ó Foighil, 2004; Mathews, 2006; Calvo et al., 2009). Molecular studies on many marine organisms (including Cnidaria, Mollusca, Echinodermata and Tunicata; Dawson and Jacobs, 2001; Tarjuelo et al., 2001; Boissin et al., 2011; Lindsay and Valdés, 2016) have frequently highlighted the occurrence of cryptic species (i.e. species that cannot be distinguished based on morphology) within widely distributed species. For example, molecular data revealed that several species once considered to be widespread across the Atlantic and the Mediterranean were actually a complex of cryptic species, many of these endemic to the Mediterranean (e.g. Borsa, 2002; Calvo et al., 2009; Claremont et al., 2011; Barco et al., 2013). A high level of endemism and the presence of numerous cryptic species have been recently revealed for opisthobranchs (Carmona et al., 2011; Furfaro et al., 2016a, 2016b),

suggesting that the marine diversity in this group still needs further assessment. However, molecular studies on many Mediterranean opisthobranchs are still scarce. Therefore, in such organisms the discovery of cryptic diversity following the application of molecular tools may be anticipated.

In this study, we focused on the systematics of Mediterranean Flabellinidae and Piseinotecidae aeolid nudibranchs (Opisthobranchia) by using molecular and morphological data. Flabellinidae Bergh, 1889 is one of the largest families of the clade Aeolidida and, according to WoRMS (Bouchet, 2011), comprises 74 species in six genera: *Flabellina* Gray, 1833 (62 species), *Babakina* Roller, 1973 (4 species), *Calmella* Eliot, 1910 (2 species), *Chlamylla* Bergh, 1886 (2 species), *Cumanotus* Odhner, 1907 (3 species) and *Tularia* Burn, 1966 (1 species). Two flabellinid genera occur in the Mediterranean Sea: *Flabellina*, with nine species [including the allochthonous species *F. rubrolineata* (O’Donoghue, 1929)], and *Calmella* Eliot, 1910 with *C. cavolini* Verany (1846). The Mediterranean ‘violet’ *Flabellina* species [*F. affinis* (Gmelin, 1791), *F. ischitana* Hirano and Thompson, 1990, *F. pedata* (Montagu, 1815) (Pola et al., 2015)], *F. babai* Schmekel, 1972 and *F. lineata* (Loven, 1846) are distributed from the eastern Mediterranean basin to the

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eastern Atlantic waters, whereas *F. baetica* Garcia-Gomez, 1984, *F. confusa* González-Duarte et al., 2008 and *F. insolita* Garcia-Gomez and Cervera, 1989 are restricted to the Strait of Gibraltar. Piseinotecidae Edmunds, 1970 is a monotypic family with six species included in the genus *Piseinotectus* Er. Marcus, 1955. Among them, four are distributed in the Mediterranean Sea and in the eastern Atlantic Ocean: *P. gabinierei* (Vicente, 1975), *P. gaditanus* Cervera, García-Gómez and García, 1987, *P. sphaeriferus* (Schmekel, 1965) and the recently described *P. soussi* Tamsouri et al., 2014.

The few molecular studies on Mediterranean aeolid nudibranchs concern species from the Alboran Sea (Carmona et al., 2013), whereas a number of taxa from Pacific and Atlantic Oceans have been studied. A molecular systematics approach to the Mediterranean aeolids would be particularly fruitful for at least two reasons. First, because the presence of cryptic species has been revealed by using molecular tools (e. g. *Caloria elegans*/*Facelina quatrefagesi* reported by Furfaro et al., 2016a), suggesting that the Mediterranean species richness is still underestimated. Second, the significance of some diagnostic morphological characters used for the systematics of Flabellinidae and Piseinotecidae is questionable. For example, members of Flabellinidae are characterized by the presence of a triseriate radula and some peculiar external characters like pedunculate cerata and oral tentacles with the same length as the rhinophores. However, there are some *Piseinotectus* species that are externally very similar to a typical ‘flabellinid’ with their pedunculate cerata, but they also have a distinctive monoseriate radula (Edmunds, 2015).

In this study we used DNA sequence data from two mitochondrial (cytochrome oxidase I and 16S rRNA, COI and 16S respectively) and one nuclear (Histone 3, H3) marker from 24 aeolid taxa and we applied species delimitation analyses and phylogenetic methods based on Maximum Likelihood and Bayesian inference. The main aims of the present work are to: (i) assess the taxonomy of these species, by analysing the intraspecific v. interspecific genetic differentiation, in particular between Atlantic and Mediterranean populations, in order to reveal potential cryptic species; (ii) estimate the phylogenetic relationships among Mediterranean Flabellinidae and Piseinotecidae of the genera *Flabellina*, *Calmella* and *Piseinotectus* in order to assess whether the Mediterranean taxa included in these families and genera form monophyletic assemblages; (iii) provide a molecular assessment of the systematics of the Mediterranean Flabellinidae and discuss the reliability of commonly-used diagnostic morphological characters.

2. Materials and methods

2.1. Molecular analyses

Specimens of Flabellinidae and Piseinotecidae from different Mediterranean and eastern Atlantic localities were collected by SCUBA diving. Each specimen was photographed, measured, preserved in 95% ethanol (EtOH) and deposited as voucher at the Department of Biology and Biotechnologies ‘Charles Darwin’ (Sapienza, Rome University). *Tritonia striata* Haefelfinger, 1963 was used as the out-group because of its basal placement within Cladobranchia (following Pola and Gosliner, 2010) (Table 1).

DNA was extracted by using a standard phenol/chloroform method with EtOH precipitation as reported in Oliverio and Mariottini (2001). Amplifications were performed by PCR using universal primers: 16Sar-L and 16Sbr-H (Palumbi et al., 2001) for the 16S fragment, LCO1490 and HCO2198 (Folmer et al., 1994) for the barcode fragment of the COI, and H3AD-F and H3BD-R (Colgan et al., 1998) for the nuclear H3 marker. PCR conditions were the same for the three molecular markers and included: 5 min of initial DNA denaturation step at 94 °C; 35 cycles of 94 °C/30 s (DNA denaturation step), 46–50 °C/60 s (annealing step), 72 °C/60 s (elongation step); and 7 min of final extension at 72 °C (Furfaro et al., 2016c). All amplicons were sequenced either at the Genchiron Centre of Sequencing, ENEA (La Casaccia, Rome, Italy) or at

the European Division of Macrogen Inc. (Amsterdam, The Netherlands).

Sequences from each DNA strain were assembled and edited with Staden Package 2.0.0b9 (Staden et al., 2000). Consensus sequences of each individual were aligned together with GenBank sequences using the Muscle algorithm implemented in MEGA 6.0 (Tamura et al., 2013).

We used Gblocks 0.91b (Castresana, 2000; Talavera and Castresana, 2007) to eliminate poorly aligned positions or hyper-divergent regions of the multiple sequence alignment of the 16S rDNA. We used less stringent options such as ‘allow gap positions (with half) within the final blocks’. For each gene alignment the best evolutionary model was selected in JModel Test 0.1 (Posada, 2008) according to the Bayesian Information Criterion (BIC).

2.1.1. Species delimitation analyses

All the specimens analysed were first classified according to their morphology. These Preliminary Species Hypothesis (PSH) were subsequently assessed by molecular species identification analyses based on genetic distance and phylogenetic methods applied to the COI dataset, because this mitochondrial marker is the most useful barcode for delimiting species in this group (Pola and Gosliner, 2010). As regards taxa for which only GenBank data were available (thus a morphological assessment was not possible), we relied only on molecular species identification analyses (Table 1). As regards species identification analyses based on genetic distance, we employed the Automatic Barcode Gap Discovery (ABGD, available at <http://www.wabi.snv.jussieu.fr/public/abgd/>) to detect the so-called ‘barcode gap’ in the distribution of pairwise distances calculated in a sequence alignment (Puillandre et al., 2012a, 2012b). The ABGD analysis was performed on the ingroup dataset using the Kimura two-parameter (K2p) model and the following settings: a prior for the maximum value of intraspecific divergence between 0.001 and 0.1, 30 recursive steps within the primary partitions defined by the first estimated gap, and a gap width of 0.1. Species hypothesis were also tested using Species Identifier (Meier et al., 2006) to calculate maximum intraspecific and minimum interspecific distances (*p*-distance) within the COI dataset and to group sequences into clusters based on the pairwise distances.

Finally, we inferred putative species boundaries using the Poisson Tree Process (PTP) phylogenetic method as implemented in the bPTP web server (Zhang et al., 2013). The bPTP analysis was run with default parameters based on the BI COI tree obtained with MrBayes.

2.1.2. Phylogenetic analyses

Single gene and multilocus concatenated datasets (the mitochondrial dataset and the mitochondrial + nuclear dataset) were analysed using Bayesian Inference (BI) and Maximum likelihood (ML) methods implementing the models selected by JModel Test for each gene. All the analyses were performed either including or excluding *F. babai* since this taxon showed a variable placement in previous phylogenetic studies (Carmona et al., 2013) suggesting that it may represent a so-called ‘rogue taxon’, that is an unstable taxon lowering tree support values (Aberer et al., 2013; Agorreta et al., 2013).

BI analyses were carried out with MrBayes 3.2.6 (Ronquist et al., 2012) with four markov-chains of five million generations each, sampled every 1000 generations. Consensus trees were calculated on trees sampled after a burnin of 25%. ML searches were performed using GARLI 0.96 (Zwickl, 2006) with two independent replicates and a starting tree topology generated by a ML stepwise-addition algorithm. Nodal support was assessed by means of 1000 bootstrap replicates.

In addition to the concatenation approach, we also performed a multilocus phylogenetic analysis based on the Bayesian species tree approach implemented in the *BEAST extension of BEAST v1.8.2 (Drummond and Rambaut, 2007). This method allows taking into account the stochastic sorting of lineages in each gene tree and is based on the multi-species coalescent model (Heled and Drummond, 2010). Species were defined according to results of the morphological assessment and molecular species delimitation analyses. We used the BEAUti

Table 1

Species names, vouchers, collection localities, GenBank accession numbers and references of sequences of Flabellinidae, Piseinotecidae and the out-group taxa analysed in this study. Individuals used for concatenated analyses are indicated with * symbol.

Species	Code/Voucher	Locality	Accession numbers			References
			H3	16S	COI	
<i>Calmella cavolini</i>	MNCN15.05/53688	Otranto, Italy (MED)	–	HQ616737	HQ616772	Carmona et al. (2011)
<i>Calmella cavolini</i> *	BAU2802	Sant'Agostino, Italy (MED)	LT718577	LT718525	LT718551	Present study
<i>Calmella cavolini</i> *	BAU2803	Split, Croazia (MED)	LT718578	LT718526	LT718552	Present study
<i>Flabellina affinis</i> *	MNCN15.05/53696	Balearic Is. Menorca, Spain (MED)	HQ616782	HQ616716	HQ616753	Carmona et al. (2011)
<i>Flabellina affinis</i> *	BAU2804	Giannutri Is. Italy (MED)	LT718579	LT718527	LT718553	Present study
<i>Flabellina affinis</i> *	BAU2805	Giannutri Is. Italy (MED)	LT718580	LT718528	LT718554	Present study
<i>Flabellina affinis</i> *	BAU2806	Ponza Is. Italy (MED)	LT718581	LT718529	LT718555	Present study
<i>Flabellina affinis</i> *	BAU2807	Ponza Is. Italy (MED)	LT718582	LT718530	LT718556	Present study
<i>Flabellina affinis</i> *	BAU2808	Lefkada, Greece (MED)	LT718583	LT718531	LT718557	Present study
<i>Flabellina affinis</i> *	BAU2809	Cadiz, Spain (ATL)	LT718584	LT718532	LT718558	Present study
<i>Flabellina albomaculata</i> *	MNCN:15.05/69896	Capo Verde (E-ATL)	KJ721524	KJ721520	KJ721522	Pola et al. (2015)
<i>Flabellina albomaculata</i>	MNCN:15.05/60115	Capo Verde (E-ATL)	KJ721523	–	KJ721521	Pola et al. (2015)
<i>Flabellina amabilis</i>	–	Ross Sea (ANT)	–	–	GQ292022	Shields et al. (2009, unpublished)
<i>Flabellina athadona</i>	–	South Korea (NW-PAC)	–	–	KT724949	Jung and Park (2015)
<i>Flabellina babai</i> *	MNCN15.05/53698	Chafarinas Is. Spain (MED)	HQ616783	HQ616717	HQ616754	Carmona et al. (2011)
<i>Flabellina babai</i> *	BAU2941	Tor Paterno, Italy (MED)	LT718585	LT718533	LT718559	Present study
<i>Flabellina babai</i> *	BAU2942	Giannutri Is. Italy (MED)	LT718586	LT718534	LT718560	Present study
<i>Flabellina baetica</i> *	MNCN15.05/53699	WAndalusia, Spain (ATL)	HQ616784	HQ616718	HQ616755	Carmona et al. (2011)
<i>Flabellina cacatoica</i>	MCN/ADN51922	–	–	–	XJ087528	Carmona et al. (2013)
<i>Flabellina confusa</i> *	MNCN/ADN51998	Arcachon, France (ATL)	JX087627	JX087491	JX087557	Carmona et al. (2013)
<i>Flabellina confusa</i> *	MNCN/ADN51999	Arcachon, France (ATL)	JX087626	JX087490	JX087556	Carmona et al. (2013)
<i>Flabellina exoptata</i> *	LACM153895	–	JQ699397	JQ699485	JQ699572	Churchill et al. (2013, unpublished)
<i>Flabellina fusca</i> *	LACM172497	–	JQ699398	JQ699486	JQ699573	Churchill et al. (2013, unpublished)
<i>Flabellina gracilis</i> *	BAU2810	Gulen, Norway, Norwegian Sea (NE-ATL)	LT718587	LT718535	LT718561	Present study
<i>Flabellina gracilis</i> *	BAU2811	Gulen, Norway, Norwegian Sea (NE-ATL)	LT718588	LT718536	LT718562	Present study
<i>Flabellina gracilis</i>	MT09706	North Sea (NE-ATL)	–	–	KR084560	Barco et al. (2016)
<i>Flabellina gracilis</i>	MT09705	North Sea (NE-ATL)	–	–	KR084492	Barco et al. (2016)
<i>Flabellina gracilis</i>	MT09694	North Sea (NE-ATL)	–	–	KR084727	Barco et al. (2016)
<i>Flabellina gracilis</i>	MT09704	North Sea (NE-ATL)	–	–	KR084774	Barco et al. (2016)
<i>Flabellina ischitana</i> ATL *	BAU2812	Cadiz, Spain (E-ATL)	LT718589	LT718537	LT718563	Present study
<i>Flabellina ischitana</i> ATL *	MNCN15.05/53700	Temara, Morocco (E-ATL)	HQ616785	HQ616719	HQ616756	Carmona et al. (2011)
<i>Flabellina ischitana</i> ATL *	MNCN15.05/53697	WAndalusia, Spain (E-ATL)	HQ616786	HQ616720	HQ616757	Carmona et al. (2011)
<i>Flabellina ischitana</i> MED *	BAU2813	Lefkada, Greece (MED)	LT718590	LT718538	LT718564	Present study
<i>Flabellina ischitana</i> MED *	BAU2815	Argentario, Italy (MED)	LT718591	LT718539	LT718565	Present study
<i>Flabellina ischitana</i> MED *	BAU2816	Tor Paterno Italy MED	LT718592	LT718540	LT718566	Present study
<i>Flabellina lineata</i> *	BAU2817	Le Formiche Is. GR Italy (MED)	LT718593	LT718541	LT718567	Present study
<i>Flabellina lineata</i> *	BAU2818	Tor Paterno, Italy (MED)	LT718594	LT718542	LT718568	Present study
<i>Flabellina lineata</i> *	BAU2819	Swedish west coast (NE-ATL)	LT718595	LT718543	LT718569	Present study
<i>Flabellina lineata</i> *	BAU2820	Swedish west coast (NE-ATL)	LT718596	LT718544	LT718570	Present study
<i>Flabellina pedata</i> *	MNCN15.05/53702	Malaga, Spain (MED)	HQ616787	HQ616721	HQ616758	Carmona et al. (2011)
<i>Flabellina pedata</i> *	BAU2821	Ljubač, Croatia (MED)	LT718597	LT718545	LT718571	Present study
<i>Flabellina pedata</i> *	BAU2823	Circeo, Italy (MED)	LT718598	LT718546	LT718572	Present study
<i>Flabellina pedata</i> *	BAU2824	Tremiti, Italy (MED)	LT718599	LT718547	LT718573	Present study
<i>Flabellina pedata</i>	MT09669	North Sea (NE-ATL)	–	–	KR084950	Barco et al. (2016)
<i>Flabellina pedata</i>	MT09670	North Sea (NE-ATL)	–	–	KR084903	Barco et al. (2016)
<i>Flabellina pedata</i>	MT09680	North Sea (NE-ATL)	–	–	KR084869	Barco et al. (2016)
<i>Flabellina pedata</i>	MT09671	North Sea (NE-ATL)	–	–	KR084838	Barco et al. (2016)
<i>Flabellina pedata</i>	MT09672	North Sea (NE-ATL)	–	–	KR084588	Barco et al. (2016)
<i>Flabellina pedata</i>	MT09679	North Sea (NE-ATL)	–	–	KR084504	Barco et al. (2016)
<i>Flabellina salmonacea</i>	HLC30316	Canada: Nunavut, Devon Is., Cape Riley (NW-ATL)	–	–	KF643961	Layton et al. (2014)
<i>Flabellina salmonacea</i>	HLC30139	Canada: Nunavut, Igloolik (NW-ATL)	–	–	KF643917	Layton et al. (2014)
<i>Flabellina salmonacea</i>	11 BFMOL0065	Canada: New Brunswick, St. Andrews, (NW-ATL)	–	–	KF643407	Layton et al. (2014)
<i>Flabellina salmonacea</i>	HLC30136	Canada: Nunavut, Igloolik (NW-ATL)	–	–	KF643282	Layton et al. (2014)
<i>Flabellina salmonacea</i>	BIOUCANHLC25326	Canada: Nunavut, Igloolik (NW-ATL)	–	–	GU802423	Layton et al. (2014)
<i>Flabellina</i> sp.	PPM193	Canada: Manitoba, Churchill, Hudson Bay (NW-ATL)	–	–	KF643452	Layton et al. (2014)
<i>Flabellina</i> sp.	PPM197	Canada: Manitoba, Churchill, Hudson Bay (NW-ATL)	–	–	KF643409	Layton et al. (2014)
<i>Flabellina trilineata</i> *	–	–	JQ699399	JQ699487	–	Churchill et al. (2013, unpublished)
<i>Flabellina trilineata</i>	–	Ross Sea (ANT)	–	–	GQ292024	Shields et al. (2009, unpublished)
<i>Flabellina trophina</i>	–	Ross Sea (ANT)	–	–	GQ292023	Shields et al. (2009, unpublished)
<i>Flabellina</i> cfr. <i>trophina</i>	11BIOAK0013	USA: Alaska, Cook Inlet (NE-PAC)	–	–	KF644188	Layton et al. (2014)
<i>Flabellina verrucosa</i>	MT09707	North Sea (NE-ATL)	–	–	KR084722	Barco et al. (2016)
<i>Flabellina verrucosa</i>	11BIOAK-0554	USA: Alaska, Cook Inlet, China Poot (NE-PAC)	–	–	KF644335	Layton et al. (2014)
<i>Flabellina verrucosa</i>	10BCMOL00319	Canada: British Columbia, Haida Gwaii (NE-PAC)	–	–	KF644243	Layton et al. (2014)

(continued on next page)

Table 1 (continued)

Species	Code/Voucher	Locality	Accession numbers			References
			H3	16S	COI	
<i>Flabellina verrucosa</i>	11BIOAK0165	USA: Alaska, Cook Inlet, McDonald Spit (NE-PAC)	–	–	KF644184	Layton et al. (2014)
<i>Flabellina verrucosa</i>	10BCMOL00435	Canada: British Columbia, Bamfield (NE-PAC)	–	–	KF643967	Layton et al. (2014)
<i>Flabellina verrucosa</i>	10BCMOL00428	Canada: British Columbia, Bamfield(NE-PAC)	–	–	KF643902	Layton et al. (2014)
<i>Flabellina verrucosa</i>	11BIOAK0164	USA: Alaska, Cook Inlet, McDonald Spit (NE-PAC)	–	–	KF643898	Layton et al. (2014)
<i>Flabellina verrucosa</i>	10BCMOL00429	Canada: British Columbia, Bamfield (NE-PAC)	–	–	KF643853	Layton et al. (2014)
<i>Flabellina verrucosa</i>	11BIOAK0012	USA: Alaska, Cook Inlet, McDonald Spit (NE-PAC)	–	–	KF643647	Layton et al. (2014)
<i>Flabellina verrucosa</i>		USA, (NW-ATL)	–	AF249245	AF249790	Wollscheid-Lengeling et al. (2001)
<i>Flabellina verrucosa</i>	Individual 14	Lunnevik, Sweden (NE-ATL)	–	–	AB180829	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 13	Lunnevik, Sweden (NE-ATL)	–	–	AB180828	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 11	Lunnevik, Sweden (NE-ATL)	–	–	AB180827	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 10	Lunnevik, Sweden (NE-ATL)	–	–	AB180826	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 9	Yttre Vattenholmen, Sweden (NE-ATL)	–	–	AB180825	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 8	Yttre Vattenholmen, Sweden (NE-ATL)	–	–	AB180824	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 7	Yttre Vattenholmen, Sweden (NE-ATL)	–	–	AB180823	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 6	Yttre Vattenholmen, Sweden (NE-ATL)	–	–	AB180822	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 5	Yttre Vattenholmen, Sweden (NE-ATL)	–	–	AB180821	Eriksson et al. (2006)
<i>Flabellina verrucosa</i>	Individual 4	Yttre Vattenholmen, Sweden (NE-ATL)	–	–	AB180820	Eriksson et al. (2006)
<i>Piseinotectus gabinierei</i> *	MNCN/ADN52000	Andalusia, Spain (MED)	JX087631	JX087495	JX087561	Carmona et al. (2013)
<i>Piseinotectus gabinierei</i> *	BAU2825	Giannutri Is. Italy (MED)	LT718600	LT718548	LT718574	Present study
<i>Piseinotectus gaditanus</i> *	MNCN15.05/53704	WAndalusia, Spain (E-ATL)	HQ616788	HQ616722	HQ616759	Carmona et al. (2013)
<i>Piseinotectus soussi</i> *	BAU2952	WAndalusia, Spain (ATL)	LT718601	LT718549	LT718575	Present study
<i>Piseinotectus soussi</i> *	BAU2953	WAndalusia, Spain (ATL)	LT718602	LT718550	LT718576	Present study
<i>Facelina quatrefagesi</i> *	BAU2697	Tor Paterno, Italy (MED)	LT596563	LT596544	LT596552	Furfaro et al. (2016a)
<i>Facelina quatrefagesi</i> *	BAU2698	Tor Paterno, Italy (MED)	LT596564	LT596545	LT596553	Furfaro et al. (2016a)
<i>Tritonia striata</i> *	BAU2695	Giannutri Is., Tuscany, Italy (MED)	LT615407	LT596542	LT596540	Furfaro et al. (2016a)
<i>Tritonia striata</i> *	BAU2696	Le Formiche Is., Grosseto, Italy	LT615408	LT596543	LT596541	Furfaro et al. (2016a)

utility to implement a lognormal relaxed clock model, the best model of nucleotide substitution for each gene, a Yule process of speciation as tree prior, and a random starting tree. We linked the tree models for the COI and 16S mitochondrial genes, because they are genetically linked. Two independent runs of 100 million generations each, sampling every 10,000 generations were performed. We used Tracer v1.6 (Drummond and Rambaut, 2007) to check for runs' mixing and convergence, LogCombiner to combine runs' output, and TreeAnnotator to summarize the trees and calculate nodes' posterior probability (burnin = 25%).

2.2. Morphological analyses

We performed a morphological assessment of taxonomic characters distinguishing Flabellinidae from Piseinotectidae. The buccal apparatus of *F. confusa* (n = 3), *P. gaditanus* (n = 2) and *P. gabinierei* (n = 1) was analysed by using both optical and Scanning Electronical Microscopes (SEM). The radula of specimens of *C. cavolini* and *F. ischitana* was also analysed for a comparison with the radula of other *Flabellina* species. Buccal masses were removed and dissolved in a 10% NaOH solution for radulae extraction. Radulae and jaws were rinsed in water, dried, and mounted for examination by optical microscopy as reported in Furfaro et al. (2016c). To obtain high resolution SEM images, dissected radulae were dehydrated in 100% EtOH through a graded series (50-70-90-95-100% EtOH), critical point-dried in a Balzer Union CPD 030 unit, gold coated in an Emitech K550 unit, and finally examined by using the field emission SEM column of the Dualbeam (FIB/SEM) Helios Nanolab (FEI Company, Eindhoven, The Netherlands) at the LIME (Electron Microscopy Interdepartmental Laboratory, Roma Tre University), with secondary electrons and an operating voltage of 5 kV.

3. Results

3.1. Molecular datasets

We obtained 78 sequences from 23 Flabellinidae and 3 Piseinotectidae specimens collected from 15 Mediterranean and eastern Atlantic localities. Additional sequences, mainly from Mediterranean and Atlantic specimens, were retrieved from GenBank (Table 1) with a definitive dataset consisting of 179 sequences. Multiple sequence alignments included 294 positions and 48 sequences for the H3, 581 positions and 86 sequences for the COI and 383 positions and 49 sequences for the 16S. Multilocus datasets included only taxa for which sequences of at least three genes were available (41 taxa).

3.1.1. Species delimitation analyses

All the species delimitation analyses confirmed the PSH based on the morphological identification, except for *F. ischitana* and *F. gracilis* which resulted in two different species hypothesis each. Interestingly, the two clades of *F. ischitana* included Mediterranean and Atlantic specimens, respectively. Minimum intra-clade COI *p*-distances within *F. ischitana* and *F. gracilis* was 12.9% and 12.5%, respectively. Moreover, species delimitation analyses highlighted two divergent clades within *F. trophina* and *F. verrucosa* based on GenBank data, with 18.9% and 20.1% of minimum intra-clade COI *p*-distances, respectively.

Results obtained from the ABGD analysis (Fig. 1A) were congruent with those obtained with bPTP and Species identifier. The 30 steps (K2p) returned in 24 PSH (Preliminary Species Hypothesis). The bPTP analysis resulted in the same species hypothesis with all groups receiving a Bayesian posterior value > 95% except for *F. affinis* (Bpp = 0.89), *F. confusa*/*P. gaditanus* (Bpp = 0.86), *F. gracilis* (Bpp = 0.88) and *F. salmonacea* (Bpp = 0.51) (Fig. 1B). The Species Identifier analysis based on the minimum interspecific and the maximum intraspecific COI *p*-distances (Fig. 1C) grouped the individuals in 24 different clusters again with the four species complexes split into

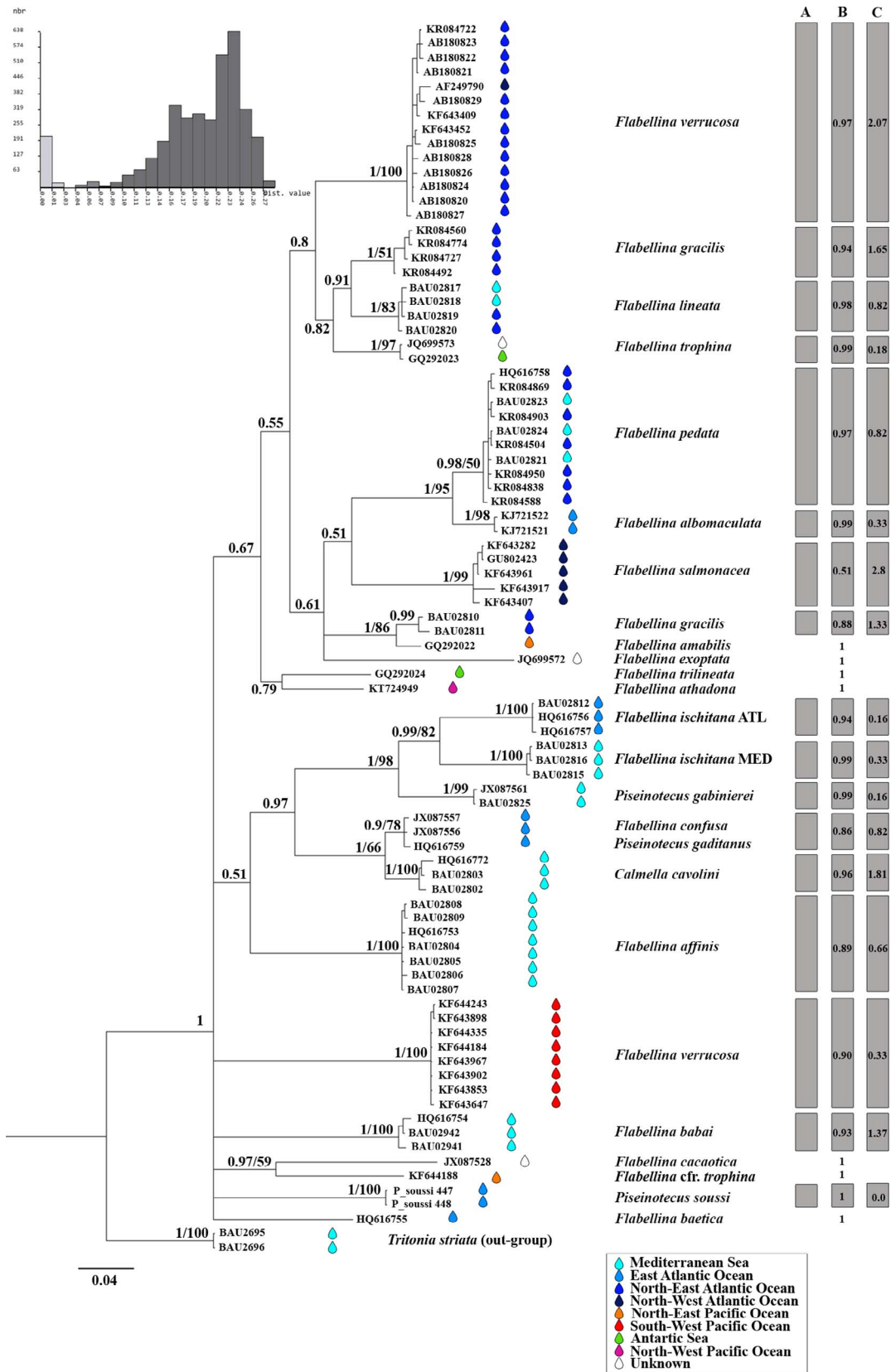


Fig. 1. Bayesian inference tree based on the COI sequence dataset along with results from species-delimitation analyses. Numbers at nodes indicate Bayesian Posterior probability (BPP; left) and bootstrap support from the Maximum-Likelihood analysis (BP; right). BPP < 0.70 and BS < 50% are not reported. The histogram shows the distribution of the pairwise genetic distances (K2p) in intraspecific (left, light grey) and interspecific (right, dark grey) comparisons. (A) ABGD Species Delimitation Hypothesis. (B) PTP results with Bayesian support values. (C) Species Identifier with the maximum intraspecific divergence values indicated as percentages.

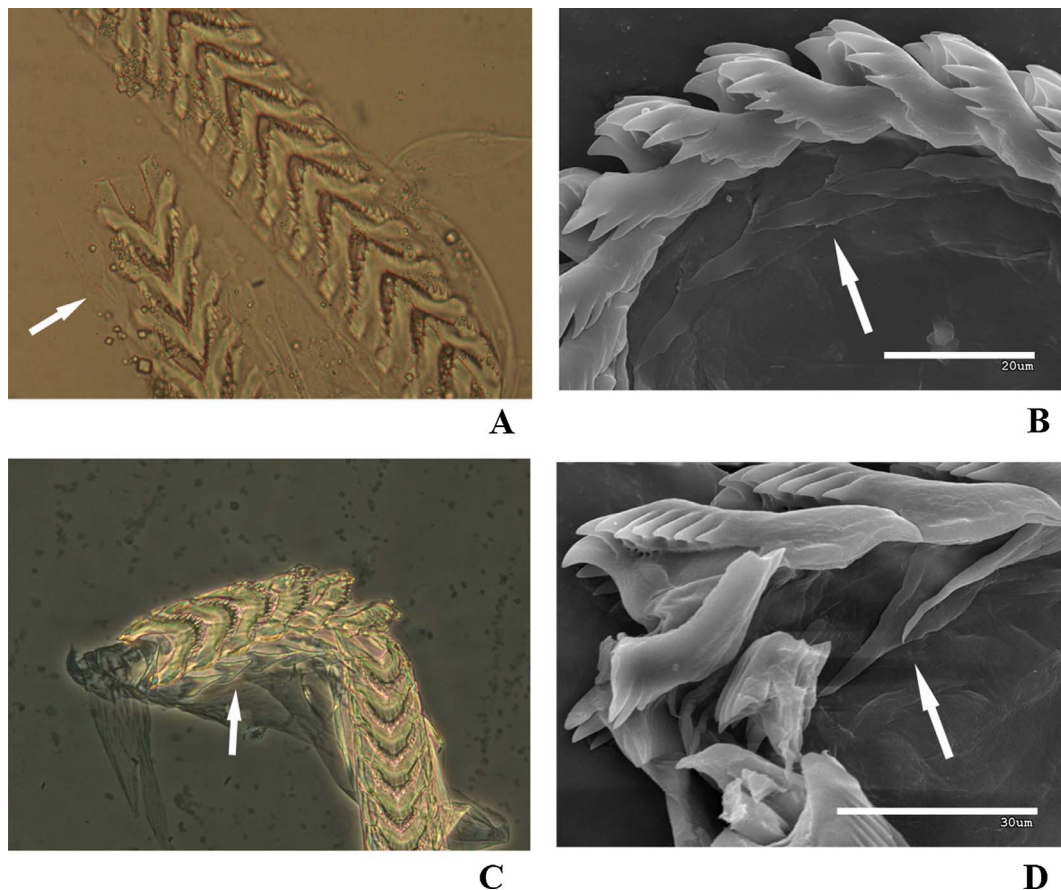


Fig. 3. Morphological analyses of the radular apparatus of *Flabellina confusa*/*Piseinotectus gaditanus* (A, B) and *Piseinotectus gabinierei* (C, D) performed using both SEM (B, D) and optical microscope (A, C). Scale bars 20 µm (B) and 30 µm (D). Arrows highlight the lateral teeth.

3.2. Morphological analyses

Morphological analyses of the buccal apparatus of *F. confusa*, *P. gaditanus* and *P. gabinierei* performed using both SEM and optical microscopes confirmed the presence of a triseriate radula in *F. confusa* and revealed, for the first time, the presence of the lateral teeth also in *P. gaditanus* and *P. gabinierei* (Fig. 3A–D). In all the analysed species, the lateral teeth were smooth and without any denticles on their margins. These lateral teeth appeared vestigial and generally were barely visible (Fig. 3). Moreover, the radula from *C. cavolini* was observed with SEM and optical microscopy definitively assessing it is triseriate and with straight lateral teeth (Fig. 4A, B). The radula from Mediterranean specimens of *F. ischitana* was also confirmed as triseriate, but showing lateral teeth that can be smooth or with denticles increasing in number from the older region of the radula to the younger. (Fig. 4C, D).

4. Discussion

4.1. Sibling species in the *Flabellinidae*

Some aeolid species of the genus *Flabellina* are distributed both in the Mediterranean Sea and in the eastern Atlantic Ocean. Our COI dataset allowed assessing the genetic differentiation at this barcoder marker between Mediterranean and Atlantic populations ascribed to *F. ischitana*, *F. lineata* and *F. pedata* (see Table 1) and also between populations of *F. gracilis*, *F. trophina* and *F. verrucosa*.

While molecular analyses of the two populations of *F. lineata* and *F. pedata* showed a COI genetic divergence (maximum intraspecific COI *p*-distance of 0.82% for both species) that is comprised within the intraspecific range of variation reported for molluscs in general (Hebert

et al., 2003) and for heterobranchs (Wägele et al., 2010; Carmona et al., 2011, 2013; Jörger et al., 2012; Padula et al., 2014) (Fig. 1). Unexpectedly high values of genetic distance were observed between Mediterranean and Atlantic populations of *F. ischitana*. Individuals morphologically identified as *F. ischitana* formed two well supported clades in all phylogenetic trees and showed a minimum COI *p*-distance of 12.5%. These results suggest that Mediterranean and Atlantic populations of *F. ischitana* may represent indeed two ‘cryptic species’. Here, we provisionally named these two taxa as the “Mediterranean” and the “Atlantic” *F. ischitana* (MED and ATL in the figures, respectively), but according to the type locality of the species (Ischia Island, Mediterranean Sea) *F. ischitana* can be considered as the valid name for the Mediterranean species. The description of the Atlantic form as a new species is currently in progress and will be provided in a future work.

Other possible cases of cryptic species complexes include *F. gracilis*, *F. trophina* and *F. verrucosa*. The Atlantic populations of *F. gracilis* split in two divergent clades in the phylogenetic analyses, which may well represent cryptic species. The GenBank sequences of *F. trophina* (Accession numbers GQ292023 and JQ699573 under the synonym *F. fusca*) and *F. cfr. trophina* (Accession number KF644188) showed a very high COI distances (18.9%) suggesting either a case of misidentification or of cryptic species within *F. trophina*. Finally, high values of genetic divergence were also found between individuals identified as *F. verrucosa*. The minimum COI distance between populations from the Atlantic Ocean and those from the Pacific Ocean was 20.1%. The Atlantic clade is composed by specimens from the eastern side and only one individual from the western coast (Wollscheid-Lengeling et al., 2001), suggesting a possible amphiatlantic distribution. Moreover, two GenBank sequences under the name of *Flabellina* sp. (Accession numbers KF643452 and

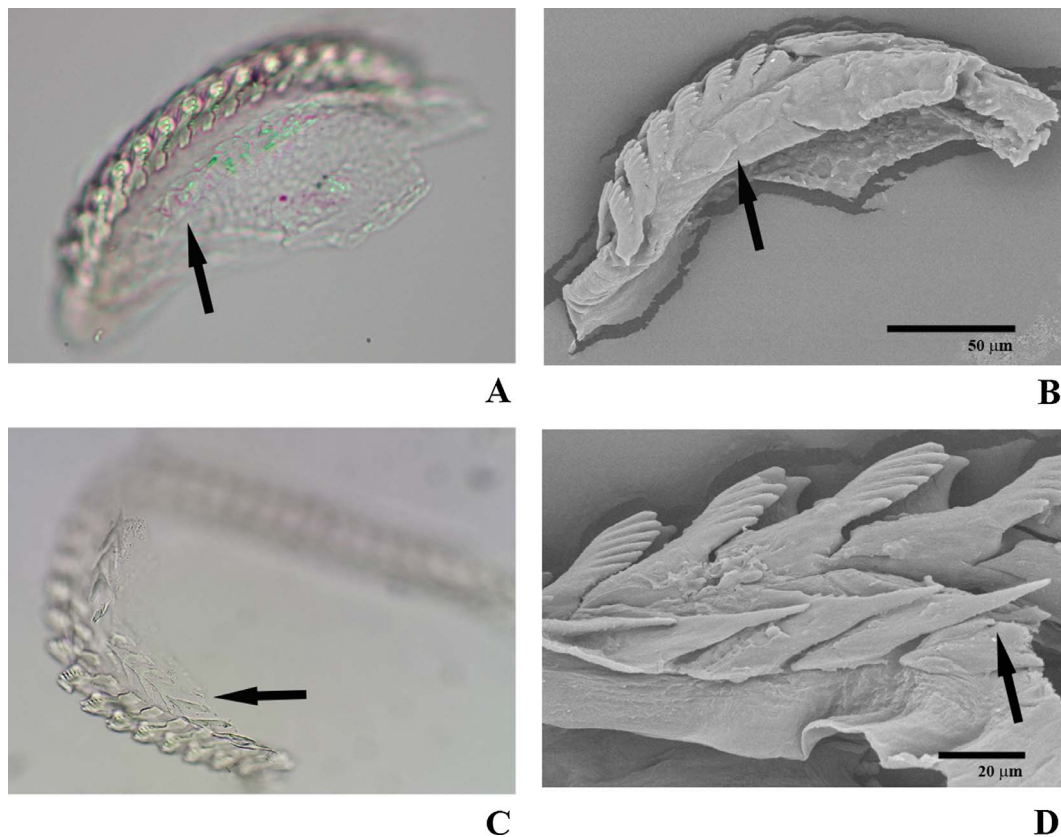


Fig. 4. Morphological analyses of the radular apparatus. (A) An entire radula of *Calmella cavolini* specimen. (B) Particular of the lateral teeth of *C. cavolini*. (C) An entire radula of Mediterranean *Flabellina ischitana* specimen. (D) Particular of the lateral teeth of *F. ischitana* performed using both SEM (B, D) and optical microscope (A, C). Scale bars 50 μm (B) and 20 μm (D). Arrows highlight the lateral teeth.

KF643409 respectively) were molecularly assigned to the Atlantic *F. verrucosa* clade.

While further studies are needed to delimit taxonomic units within these taxa, our study identified putative species complexes in most of the species for which molecular data from multiple individuals were available, suggesting a high rate of cryptic speciation within Flabellinidae. This result is consistent with the expectation of high cryptic diversity within the Mediterranean nudibranchs and claim for further molecular assessments.

4.2. Flabellinidae and Piseinotecidae: Molecular inference and morphological synapomorphies

Consistently with morphological data, our molecular analyses indicated that Mediterranean species of *Piseinotecus* - *P. gaditanus* and *P. gabinieri* - are nested within *Flabellina* clades with high support values (Fig. 2; Fig. S1), being in agreement with previous indications (Gosliner et al., 2007; Carmona et al., 2011, 2013; Edmunds, 2015) of a polyphyletic condition of Flabellinidae and Piseinotecidae families as currently conceived. Furthermore, in our phylogenetic trees *P. soussi* is sister to the flabellinid clade. This species was described as very similar to a “violet flabellinid” but with monoseriate radula (Tamsouri et al., 2014) and for this reason included in *Piseinotecus*. Further studies are required to better evaluate the taxonomic position of this species within Aeolidioidea.

Phylogenetic trees (Fig. 2, Fig. S1) showed two main clades, the “*F. pedata* and allies” clade and the “*F. affinis* and allies” clade, also supported by morphological features. The “*F. pedata* and allies” clade (BPP = 1) shows the presence of singularly attached cerata on the dorsal notum, whereas members of the second clade are characterized by the occurrence of pedunculate (grouped) cerata. Since *F. baetica*,

which is sister to the “*F. pedata* and allies” and the “*F. affinis* and allies” clades, presents single cerata on the notum (Fig. 2), it is possible to speculate that the pedunculate cerata may represent a synapomorphy of the “*F. affinis* and allies” clade. Within the “*F. pedata* and allies” clade (BPP = 1), the close relationships between *F. pedata* and the recently described *F. albomaculata* inferred based on molecular data (BPP = 1) is further corroborated by the fact that these taxa share smooth rhinophores, the pleuroproct position of the anal opening and the violet body color (Pola et al., 2015). Within the “*F. affinis* and allies” clade (BPP = 1), *F. affinis* (the type species of the genus *Flabellina*) is sister to a first clade grouping *F. ischitana* and *P. gabinieri* and a second one composed by *C. cavolini* and *F. confusa*/*P. gaditanus* (Fig. 2). Members of these two clades show a smooth condition for the lateral teeth, as opposed to the denticulate (ancestral) state found in *F. affinis* (and all other Flabellinidae), suggesting that the smooth condition might represent a new synapomorphy of these clades, with the exception of the *F. ischitana* complex, which maintains both character states. *P. gaditanus* is the sister species of *C. cavolini* with high statistical support (BPP = 1). These two species have a similar lateral teeth which are smooth and very hard to observe, as already discussed in the past by Pruvot-Fol (1953) and Ev. Marcus (1976).

Interestingly, the sister taxa *C. cavolini* and *F. confusa*/*P. gaditanus* show a 5.0% of minimum interspecific divergence (COI *p*-distance) and a very similar morphology. In fact, these two taxa differ from each other only by the absence and presence, respectively, of a few white dots on the cerata surface. Furthermore, the intraspecific divergence (COI *p*-distance) of the *C. cavolini* species is about 1.8% that indicate a quite level of variability within this species. A deeper study would be suitable to unravel the distribution of the genetic intra and interspecific divergence among these two closely related taxa.

The monotypic *Calmella* genus is nested within the *Flabellina* clade

Table 2

Scheme of the proposed systematic changes for the “*Flabellina affinis* and allies” clade. Mediterranean Sea populations of *Flabellina ischitana* maintain the name whereas Atlantic Ocean populations are assigned to a new species which is under description.

Valid Species	Synonym	Distribution
<i>Flabellina cavolini</i>	<i>Calmella cavolini</i>	Mediterranean Sea
<i>Flabellina gabinierei</i>	<i>Piseinotocus gabinierei</i>	Mediterranean Sea
<i>Flabellina gaditana</i>	<i>Flabellina confusa</i> / <i>Piseinotocus gaditanus</i>	Atlantic Ocean
<i>Flabellina</i> sp. (to be described)	<i>Flabellina ischitana</i>	Atlantic Ocean

in our molecular phylogenetic trees (Fig. 2; Fig. S1). Despite *Calmella* would be characterized by some peculiar features of the buccal apparatus according to original studies (Marcus, 1976; Vayssièrè, 1888), our morphological re-assessment supports the inclusion of this genus within *Flabellina* (see the last section). Regarding the taxonomic status of the Piseinotecidae, we could only assess that the taxa analysed form a polyphyletic assemblage. In our study we did not include the type species of the family Piseinotecidae, *P. divae*, thereby a conclusive systematic assessment of this family remains pending the study of *P. divae* and possibly additional species.

4.3. Morphology of Flabellinidae

Anatomical analyses support the revision of the classification of Flabellinidae and Piseinotecidae as supported by molecular data. Together these results highlight some incorrect species classification occurred in the past and reject the taxonomic validity of some morphological characters historically considered as diagnostic for species delimitation. Based on morphology, Flabellinidae and Piseinotecidae species are considered very closely related, but separated into two distinct families based on few diagnostic features. A triseriate or monoseriate radula is currently the character mostly used to assign species to Flabellinidae or Piseinotecidae, respectively (Tamsouri et al., 2014; Edmunds, 2015).

Results from our study clearly demonstrated a triseriate radula in the Mediterranean species *P. gaditanus* and *P. gabinierei* (Fig. 3). The assignment of these species to the family Piseinotecidae was clearly due to an erroneous interpretation of the state of this character. This is possibly due to the fact that the smooth lateral, and vestigial, teeth are somehow difficult to observe especially if using SEM, since lateral teeth can be lost during sample preparation (e.g., in the tissue digestion phase). The difficulty in detecting this anatomical character generated also the erroneous definition of the radula type of *C. cavolini* that here we demonstrated to be triseriate with smooth lateral teeth (Fig. 4A, B). The genus *Calmella* is a striking example about the misperception of this character. The original description of *C. cavolini* by Verany (1846) did not mention the shape of the radula, but Prouvot-Fol (1953) questioned about its ‘triseriate’ condition, by reporting (in a drawing) one individual having an “aberrant” triseriate radula characterized by very small, and hard to see, lateral teeth. Later on, Schmekel (1965) and Schulze and Wägele (1998) stated that the radula of this species was monoseriate. To make the systematics of this taxon even more confused, the individual depicted by Prouvot-Fol (1953) appears morphologically identical to *F. confusa*, described about half a century later by González-Duarte et al. (2008), which has a triseriate radula. It is worth to point out that the original description of *F. confusa* reported this species as “morphologically indistinguishable” from *P. gaditanus* except for the triseriate radula. Morphological results of the present study provide compelling evidence that both *F. confusa* and *P. gaditanus* have triseriate radula, and thus, it is not possible to distinguish them by any morphological character.

Another anatomical character historically used for taxonomic consideration within aeolids is the position of the anus. The pleuroproct

anus (i.e. lateral to the rows of cerata) was considered as the diagnostic character of the Flabellinidae, while the acleioproct condition (i.e. anus in the interhepatic space) was diagnostic of the Piseinotecidae. Molecular and morphological results from our study clearly indicate that at least part of the species currently in the Piseinotecidae are actually to be assigned to the family Flabellinidae. Therefore, we conclude that the acleioproct or the pleuroproct condition of the anus is not a valid diagnostic character to discriminate Flabellinidae.

Finally, we found that the presence of pedunculate cerata is an important morphological synapomorphy of the ‘*Flabellina affinis* and allies’ clade as recovered by the phylogenetic analysis. This morphological character was used in the past to distinguish the families Flabellinidae and Coryphellidae Bergh, 1889 (Prouvot-Fol, 1954; Taylor and Sohl, 1962; Odhner, 1968; Vaught, 1989). The latter family in fact, would be characterized by the presence of single cerata directly inserted on the dorsal notum, and not pedunculate as in the Flabellinidae. Flabellinidae and Coryphellidae were synonymized as Flabellinidae by Marcus and Marcus (1967), and this is the most accepted systematics to date. However, according to our molecular analyses, the pedunculate cerata represents a striking diagnostic character of the monophyletic clade that include the type species of the genus *Flabellina* (*F. affinis*) and deserves special attention in future systematics revisions of the genus *Flabellina*. At this regard it is worth noticing that singularly attached cerata are present in the “*F. pedata* and allies” clade as well as in all basal taxa of our phylogenetic trees (e.g. *F. baetica*), suggesting that the single cerata is likely a plesiomorphic state of the character.

5. Concluding systematic remarks

On the basis of results from the molecular and morphological analyses of this study, we propose the following systematic changes (summarized in Table 2): (1) *F. confusa* is considered as a synonym of *P. gaditanus*, since their genetic divergence is well within the intraspecific range and the description of *P. gaditanus* radula was based on the erroneous observation of its lateral teeth; (2) *P. gaditanus* is to be moved into the family Flabellinidae and renamed as *Flabellina gaditana* comb. nov. on the base of its phylogenetic position nested within *Flabellina*, and the triseriate radula that it shares with all other Flabellinidae; (3) *P. gabinierei* and (4) *C. cavolini* are to be included in the genus *Flabellina* as *F. gabinierei* comb. nov. and *Flabellina cavolini* comb. nov., respectively, on the base of their phylogenetic position nested within the *Flabellina* clade and their triseriate radula. The genus *Calmella* (monotypic) is a synonym of *Flabellina*. Finally, we discovered a cryptic species within *F. ischitana*, which will be described elsewhere.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2017.09.001>.

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