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Not so sluggish: the success of the *Felimare picta* complex (Gastropoda, Nudibranchia) crossing Atlantic biogeographic barriers

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Abstract

The molecular phylogeny of the Atlanto-Mediterranean species of the genus Felimare, particularly those attributed to the species *F. picta*, was inferred using two mitochondrial markers (16S and COI). A recent revision of the Chromodorididae clarified the taxonomic relationships at the family level reclassifying all eastern Pacific, Atlantic and Mediterranean species of the genus *Hypselodoris* and two species of the genus *Mexichromis*, within the genus *Felimare*. However, conflicting taxonomic classifications have been proposed for a group with overlapping morphological characteristics and geographical distributions designated here as the *Felimare picta* complex. Three major groups were identified: one Mediterranean and amphi-Atlantic group; a western Atlantic group and a tropical eastern Atlantic group. *F. picta* forms a paraphyletic group since some subspecies are more closely related with taxa traditionaly classified as independent species (e.g. *F. zebra*) than with other subspecies with allopatric distributions (e.g. *F. picta picta* and *F. picta tema*). Usually, nudibranchs have adhesive demersal eggs, short planktonic larval phases and low mobility as adults unless rafting on floating materials occurs. However, the phylogeny of the *F. picta* complex suggests they had an unusual success crossing main Atlantic biogeographic barriers including the mid-Atlantic barrier. This ability to cross different biogeographic barriers may be related with *F. picta* distinct life history and ecological traits. Compared to other Chromodorididae *F. picta* presents large eggs and planktotrophic larvae which could be related with a longer planktonic phase.

Keywords: mitochondrial DNA, mid-Atlantic barrier, speciation

1. Introduction

Nudibranchs are heterobranch mollusks (Gastropoda) that comprise more than 3000 species (Willan & Coleman, 1984). The family Chromodorididae includes some of the most striking colored nudibranchs present in almost all marine habitats. These sea slugs often present defensive adaptations that include the production or incorporation of bioactive chemicals from cnidarians or sponges upon which they feed and their toxicity is advertised by conspicuous aposematic coloration (Wollscheid & Wägele, 1999; Haber et al., 2010). These toxins have captured the interest of numerous authors (Gaspar, Rodrigues & Calado, 2009; Haber et al.,

2010; Cruz, Gaspar & Calado, 2012) and represent a source of natural compounds whose biological activity is being actively prospected. Due to these characteristics chromodorids could also be good models for the study of color pattern evolution and mimicry in marine species (Gosliner & Johnson 1999).

The absence of a comprehensive phylogeny of chromodorids still raises numerous questions, generating obstacles to future investigation. Understanding the evolutionary relationships may represent a "road map" allowing the prediction of characteristics that are still undescribed and may help to disentangle genetic from environmental effects (e.g. Gaspar, Rodrigues & Calado, 2009).

The taxonomy of the chromodorid nudibranchs was originally based on morphological data (Rudman, 1984). Gosliner & Johnson (1999) revised the phylogeny of the worldwide genus *Hypselodoris*, including some of the species that are more abundant in the northeastern Atlantic and Mediterranean Sea. These revisions were followed by a number of morphological (Alejandrino & Valdés, 2006) and molecular studies (Wollscheid & Wägele, 1999; Wollscheid-Lengeling et al., 2001; Turner & Wilson, 2007; Johnson, 2011), including the description of several new species (Dacosta, Padula & Schrödl, 2010; Ortigosa & Valdés, 2012). Johnson & Gosliner (2012), based on two mitochondrial genes, proposed a new phylogenetic hypothesis of the chromodorid and reorganized its traditional taxonomy. These authors identified the Pacific Ocean as an effective biogeographic barrier, proposing the inclusion of all eastern Pacific, Atlantic and Mediterranean *Hypselodoris*, together with two species from the genus *Mexichromis* from the eastern Pacific and Caribbean, into a different genus, *Felimare*, while retaining the west/central Pacific *Hypselodoris* species within this genus. Eastern Pacific, Atlantic and Mediterranean species previously in the genera C*hromodoris* and *Glossodoris* were included within the *Felimida*. Prior to their revision, *Hypselodoris*, C*hromodoris* and *Glossodoris* were the most species rich chromodorid genera. Recently, using additional species of these genera and acruing a nuclear DNA marker, Ortigosa et al. (2014) recovered a polytomy between several Atlantic and Mediterranean *Felimida* and *Felimare* species.

1.1. Taxonomic considerations on *Felimare picta*

Within the Chromodorididae *Felimare picta* presents the wider distribution area throughout the tropical and subtropical Atlantic Ocean, including the Mediterranean Sea and the eastern Atlantic

archipelagos of Cape Verde, Canaries, Madeira and Azores (Ortea, Valdés & García-Gómez, 1996). These coastal sea slugs are one of the largest chromodorid species and vary greatly in colour and pattern, resulting in the traditional classification under many species names (e.g. *Felimare* (*Hypselodoris*) *webbi* (d'Orbigny, 1839) and *Felimare* (*Hypselodoris*) *tema* (Edmunds, 1981)). Variation also led to innacuracies in assignment to *F. picta* subspecies using external morphology or internal anatomy (Ortea, Valdés & García-Gómez, 1996; Alejandrino & Valdés, 2006). One of the cases that illustrates the ambiguous situation of this group is the changing taxonomic status of the southwest Atlantic representatives classified as subspecies (*Felimare picta lajensis*) by Troncoso, García & Urgorri (1998), as a valid species (*Felimare lajensis*) by Domínguez, García & Troncoso (2006) and then downgraded again to a subspecific level by Dacosta, Padula & Schrödl (2010). Similarly, *Felimare tema*, originally described from Ghana by Edmunds (1981) was reclassified as subspecies (*Felimare picta tema*) by Ortea, Valdés & Garcia-Gomez (1996). Color pattern similarities between *Felimare picta verdensis* and *Felimare picta tema* (sensu Ortea, Valdés & Garcia-Gomez, 1996) also raised some doubts on the validity of these subspecies. Table S1 in Supplementary Data summarizes the main taxonomic classifications and also the overlapping distribution areas of some of these subspecies.

The phylogenetic relationships of the *F. picta* complex with other taxa long recognised as distinct species, both from the east and west Atlantic, remains largely unknown. *F. picta* is the only amphi-Atlantic species of this genus, but along the east Atlantic shores and central Atlantic islands we can find several species with overlapping distributions. Along the west Atlantic shore this phylogenetic relationship is even more complex, with a close trans-isthmian phylogenetic relationship between all eastern Pacific and the Atlantic/Mediterranean *Felimare* species, together with two species previously assigned to the genus *Mexichromis* (*F. porterae* and *F. kempfi*) reported by Johnson & Gosliner (2012).

1.2. Biogeography

The ability to cross biogeographic barriers is normally restricted to highly mobile species or species that produce propagules with high potential to disperse at least during a particular phase of their ontogeny (Briggs, 1974). Aside from landmasses, long extensions of deep oceanic water and abrupt changes of physical or chemical properties of marine water can effectively restrict the

colonization potential of inshore organisms (Floeter et al., 2008; Luiz et al., 2011). Furthermore, the effectiveness of these permeable barriers may be influenced by the potential to establish new populations in the recently colonized habitats (Luiz et al., 2011).

The mid Atlantic barrier (MAB) is a gap formed by the Atlantic Ocean basin in the last 85 Myr. It spans a minimum straight-line distance of approximately 2800 km, which represents an extreme distance relative to regular larval dispersal by marine organisms (Floeter et al., 2008; Luiz et al., 2011).

Concerning molluscs alone, Vermeij (2005) cited that 30.8% (northeastern Atlantic) to 47.3% (northwestern Atlantic) of the North-Atlantic species present an amphi-Atlantic distribution. However, *F. picta* is the Atlanto-Mediterranean Chromodorididae with the wider distribution area (Ortea, Valdés & García-Gómez, 1996) and one of the few present in both margins of the Atlantic. the Mediterranean and most islands of the tropical and temperate north Atlantic, having crossed major biogeographic barriers. With such an extensive geographical distribution, several subspecies were historically assigned to *Felimare picta*. Some of these subspecies are morphologically very similar and present overlapping geographical distributions (e.g. *F. picta picta* and *F. picta webbi* cf. Ortea, Valdés & García-Gómez, 1996). A molecular phylogeny of this group would shed some light on the taxonomic status of *F. picta* subspecies and other closely related Atlanto-Mediterranean *Felimare* species.

In the present study we intend to clarify the taxonomic status of *F. picta* and its relationships with other congeneric species. Simultaneously we want to evaluate the validity of its previously proposed subspecies that currently raise numerous identification problems to many taxonomists. The molecular phylogeny will be the starting point to infer the biogeographic relationships within a group of taxa that was able to cross the main Atlantic biogeographic barriers.

2. Material and methods

2.1. Sampling

The species sampled in the present study, the geographical origin of the samples and the GenBank accession numbers are listed in Table S2 in Supplementary Data. Specimens were identified and portions of tissue were provided by a nudibranch taxonomist (Dr. Gonçalo Calado - see Gaspar, Rodrigues & Calado (2009); Coelho & Calado (2010); Haber et al. (2010); Calado & Silva (2012); Cruz, Gaspar & Calado (2012)). Extracted DNA is available from ISPA

laboratory collections. Voucher specimens of *F. lajensis* are also available from Museu de Zoologia da Universidade de São Paulo (MZSP97468). In an attempt to detect possible intraspecific variability in this species, a total of 32 *F. picta* representing samples from its entire geographical distribution area were analysed. We included samples that are separated by geographical barriers that are highly effective for coastal species (e.g. the MAB) and others that are only effective for some particular species (e.g. the Strait of Gibraltar) (see Patarnello, Volckaert & Castilho, 2007). Additional samples belonging to 13 different *Felimare* species, which represent aproximatelly half the species described for the eastern Pacific, Atlantic and Mediterranean (WoRMS, 2014), including some already available in GenBank, were also analysed for comparative purposes to provide a broader phylogenetic framework and access the overall genetic divergence within this genus.

2.2. DNA extraction, amplification and sequencing

DNA was extracted from tissue samples preserved in ethanol, using a proteinase K/SDS based protocol (Sambrook, Fritsch & Maniatis, 1989). Primers used to amplify a fragment 461 bp long from the 16S mitochondrial rDNA (16Ssar 5' CGC CTG TTT ATC AAA AAC AT 3' and 16Sbr 5' CCG GTC TGA ACT CAG ATC ACG T 3'), and a fragment 554 bp long from the COI (LCO1490 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and HCO2198 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'), are described in Palumbi et al. (1996) and Folmer et al. (1994), respectively. The primers 28SC1(F) 5' ACC CGC TGA ATT TAA GCA T 3' and 28SD3(R) 5' GAC GAT CGA TTT GCA CGT CA 3' used by Mollaret et al. (1997), Vonnemann et al. (2005) and Klussmann-Kolb et al. (2008) were also used to attempt to amplify a fragment of the nuclear 28S rDNA in these species.

PCR conditions were conducted as follows: 2 min. 95ºC followed by 35 cycles of [95ºC (30 sec.), 54ºC (30 sec.) and 72ºC (60 sec.)] for the 16S fragment; 2 min. 95ºC followed by 35 cycles of [95ºC (45 sec.), 50ºC (45 sec.) and 72ºC (60 sec.)] for the COI fragment and 4 min. 95ºC followed by 38 cycles of [94ºC (30 sec.), 52ºC (50 sec.) and 72ºC (120 sec.)] followed by 10 min. at 72°C for the 28S fragment.

PCR products were purified using microClean (MicroZone, www.microzone.co.uk), and sequenced in STABVIDA (http://www.stabvida.net/) using the same primers.

2.3. Phylogenetic analysis

In our analysis of the phylogeny of the *Felimare picta* complex we followed Johnson (2011) including as outgroup a species from the Dendrodorididae family: *Doriopsilla pelseneeri* d'Oliveira, 1895. DNA sequences were analysed and edited with CodonCode aligner (v. 3.5, CodonCode Corporation) and were aligned separately using M-Coffee (Notredame, Higgins & Heringa, 2000). Manual alignment masking was performed, by excluding loci scored as 'bad' by M-Coffee, in order to improve sign-to-noise ratio. Transitional saturation was examined by plotting transitions and transversions against sequence divergence using GTR distance and implementing Xia et al. test (Xia et al., 2003; Xia & Lemey, 2009) test of substitution saturation available in Dambe v. 5.3.108 (Xia, 2013).

Each fragment and a concatenation of both fragments were analysed using four phylogenetic inference methods: 1) maximum-parsimony (MP) with 100 heuristic searches using random additions of sequences and implementing the TBR algorithm, as implemented in PAUP 4.0b10 (Swofford, 2001); 2) minimum-evolution (ME), also implemented in PAUP with 1000 resamplings, was implemented using the best-fit model of molecular evolution chosen according the Bayesian Information Criterion as implemented in JModeltest 2.0 (Darriba et al., 2012); 3) Maximum Likelihood, as implemented in RaxML (Stamatakis, Hoover & Rougemont, 2008) and 4) Bayesian inference (BI) performed using MCMC as implemented in MrBayes v. 3.2 (Ronquist et al., 2012), with two independent runs of four Metropolis-coupled chains of four million generations each in order to estimate the posterior probability distribution. Topologies were sampled every 100 generations and a majority-rule consensus tree was estimated after discarding the first 10% samples. Convergence was verified by inspecting the average standard deviation of split frequencies and tracing likelihood throughout samples in Tracer v1.6 (Drummond et al., 2012). Both ML and BI analyses of the concatenated alignment considered two partitions for which independent parameters were estimated. For the first three phylogenetic inference methods, branch support values for each node were tested by bootstrap analysis, with 100 resamplings (Felsenstein, 1985). Net between group mean distances were calculated using Mega (Tamura et al., 2013) using Tamura-Nei distance with gamma model estimated by the composite likelihood method.

3. Results

204 *3.1. Sequence analysis*

205 The null hypothesis of congruence between the two data sets (16S and COI rDNA) was not
206 rejected (P = 0.33) by the ILD test (Farris et al., 1995). Therefore the results presented in
207 subsequent sections relate to the analysis of the concatenation of the 16S and COI rDNA
208 fragments. The combined sequence of the 16S + COI rDNA fragments resulted in an alignment
209 of 1026 base pairs. Of these, 712 characters are constant, 66 variable characters are parsimony-
210 uninformative and 248 are parsimony-informative characters. No saturation was observed for the
211 16S and COI datasets or the concatenated fragment with both sequences (P < 0.001 for all
212 combinations) (Xia et al., 2003; Xia & Lemey 2009).

213 Minimum-evolution (ME), using the best-fit model of molecular evolution chosen according the
214 Bayesian Information Criterion as implemented in JModeltest 2.0 (Darriba et al., 2012) was
215 HKY+I+G for 16S; TrN+I+G for COI and TIM3+I+G for the concatenated 16S and COI.

216 Since we were not able to amplify the COI fragment of *F. acriba*, known from the Caribbean,
217 this species is not shown in the concatenated tree with the 16S and COI fragments (Figure 1).
218 However, all phylogenetic analysis with the 16S fragment recovered this species as the sister
219 species of *F. bayeri* with very high support values (Bayesian analysis with posterior probability
220 of 1.0 and maximum parsimony with bootstrap value of 100).

221 Estimated net evolutionary divergence between species and subspecies is presented in Table 1.
222 Genetic similarities between *F. picta* collected from Mexico to the Mediterranean, including
223 samples from the Azores, which would be classified as *F. p. picta*, *F. p. azorica*, *F. p. webbi*
224 (sensu Ortea, Valdés & García-Gómez, 1996), and haplotypes shared between these putative
225 subspecies (e.g. between *F. picta picta*, *F picta webbi*, *F. picta azorica*) revealed no genetic
226 isolation. The genetic distance between these subspecies and the west African samples (0.255-
227 0.272), which would be classified as *F. p. tema* and *F. p. verdensis* (sensu Ortea, Valdés &
228 García-Gómez, 1996) is larger than the distance shown for *F. zebra* (0.185) and is similar to the
229 one shown for *F. bayeri* (0.277).

230

Table 1 – Estimates of net evolutionary divergence between groups of sequences from *F. picta* subspecies, other Atlanto-Mediterranean *Felimare* species and Indo-West Pacific *Hypselodoris* species. The number of base substitutions per site from estimation of net average between groups of sequences are shown. Standard error estimate(s) are shown above the diagonal. The *Felimare picta* complex is highlighted in bold. Analyses were conducted using the Maximum Composite Likelihood model (Tamura, Nei & Kumar, 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.426). The analysis involved 45 mtDNA sequences and all ambiguous positions were removed resulting in a total of 1124 nucleotides in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

	Chromodoris	*F. bayeri*	*F. bilineata*	*F. californensis*	*F. cantabrica*	*F. fontadraui*	*F. kempfi*	*F. lajensis*	*F. midatlantica*	*F. orsinii*	***F. picta azorica***	***F. picta picta***	***F. picta webbi***	***F. picta tema***	***F. picta verdensis***	*F. ruthae*	*F. villafranca*	*F. zebra*	*Hypsel*
Chromodoris		0,546	0,617	0,613	0,735	0,631	0,496	0,628	0,560	0,624	**0,690**	**0,702**	**0,691**	**0,565**	**0,560**	0,872	0,576	0,591	0,6
F. bayeri	1,766		0,284	0,218	0,470	0,395	0,489	0,079	0,307	0,389	**0,082**	**0,084**	**0,083**	**0,067**	**0,063**	0,582	0,370	0,082	0,6
F. bilineata	2,055	0,922		0,250	0,464	0,379	0,561	0,288	0,286	0,371	**0,289**	**0,290**	**0,273**	**0,278**	**0,269**	0,502	0,420	0,300	0,6
F. californensis	2,069	0,692	0,771		0,442	0,363	0,648	0,199	0,330	0,382	**0,197**	**0,197**	**0,192**	**0,182**	**0,179**	0,630	0,363	0,209	0,7
F. cantabrica	2,592	1,536	1,520	1,454		0,478	0,786	0,449	0,461	0,405	**0,422**	**0,432**	**0,427**	**0,444**	**0,429**	0,747	0,280	0,494	0,9
F. fontadraui	2,095	1,260	1,227	1,162	1,596		0,633	0,453	0,309	0,329	**0,481**	**0,479**	**0,480**	**0,395**	**0,381**	0,602	0,399	0,441	0,7
F. kempfi	1,660	1,611	1,865	2,211	2,752	2,217		0,519	0,586	0,653	**0,576**	**0,577**	**0,572**	**0,533**	**0,522**	0,845	0,679	0,542	0,7
F. lajensis	2,138	0,258	0,930	0,644	1,485	1,499	1,749		0,338	0,458	**0,050**	**0,050**	**0,050**	**0,074**	**0,074**	0,652	0,346	0,022	0,7
F. midatlantica	1,839	0,941	0,919	1,053	1,497	0,968	1,973	1,085		0,356	**0,362**	**0,365**	**0,370**	**0,316**	**0,320**	0,614	0,270	0,373	0,6
F. orsinii	2,044	1,194	1,175	1,206	1,373	1,059	2,208	1,489	1,147		**0,487**	**0,490**	**0,484**	**0,381**	**0,386**	0,627	0,206	0,440	0,6
F. picta azorica	**2,340**	**0,272**	**0,944**	**0,627**	**1,364**	**1,572**	**1,966**	**0,163**	**1,130**	**1,579**		**0,000**	**0,000**	**0,081**	**0,078**	**0,651**	**0,408**	**0,054**	**0,7**
F. picta picta	**2,379**	**0,277**	**0,949**	**0,628**	**1,405**	**1,564**	**1,973**	**0,165**	**1,138**	**1,591**	**0,000**		**0,000**	**0,083**	**0,080**	**0,647**	**0,412**	**0,053**	**0,7**
F. picta webbi	**2,338**	**0,272**	**0,891**	**0,617**	**1,387**	**1,569**	**1,957**	**0,164**	**1,155**	**1,569**	**0,000**	**0,000**		**0,084**	**0,080**	**0,636**	**0,404**	**0,055**	**0,7**
F. picta tema	**1,847**	**0,211**	**0,885**	**0,572**	**1,450**	**1,259**	**1,798**	**0,246**	**0,994**	**1,180**	**0,262**	**0,269**	**0,272**		**0,004**	**0,614**	**0,404**	**0,089**	**0,6**
F. picta verdensis	**1,826**	**0,200**	**0,866**	**0,570**	**1,406**	**1,207**	**1,767**	**0,250**	**1,019**	**1,218**	**0,255**	**0,259**	**0,260**	**0,008**		**0,600**	**0,397**	**0,090**	**0,6**
F. ruthae	3,040	1,936	1,691	2,161	2,538	2,027	2,945	2,218	2,051	2,156	**2,212**	**2,206**	**2,161**	**2,090**	**2,035**		0,620	0,614	0,8
F. villafranca	1,920	1,128	1,313	1,124	0,901	1,274	2,339	1,068	0,830	0,635	**1,269**	**1,283**	**1,256**	**1,263**	**1,247**	2,112		0,362	0,6
F. zebra	1,942	0,258	0,943	0,650	1,617	1,441	1,841	0,072	1,192	1,388	**0,181**	**0,179**	**0,185**	**0,281**	**0,287**	2,045	1,100		0,7
Hypselodoris	2,209	2,135	2,113	2,736	3,266	2,459	2,473	2,693	2,350	2,401	**2,585**	**2,577**	**2,484**	**2,423**	**2,424**	2,978	2,182	2,463	

243

244 *2. Phylogenetic analysis*

245 The genus Felimare and higher order relationships

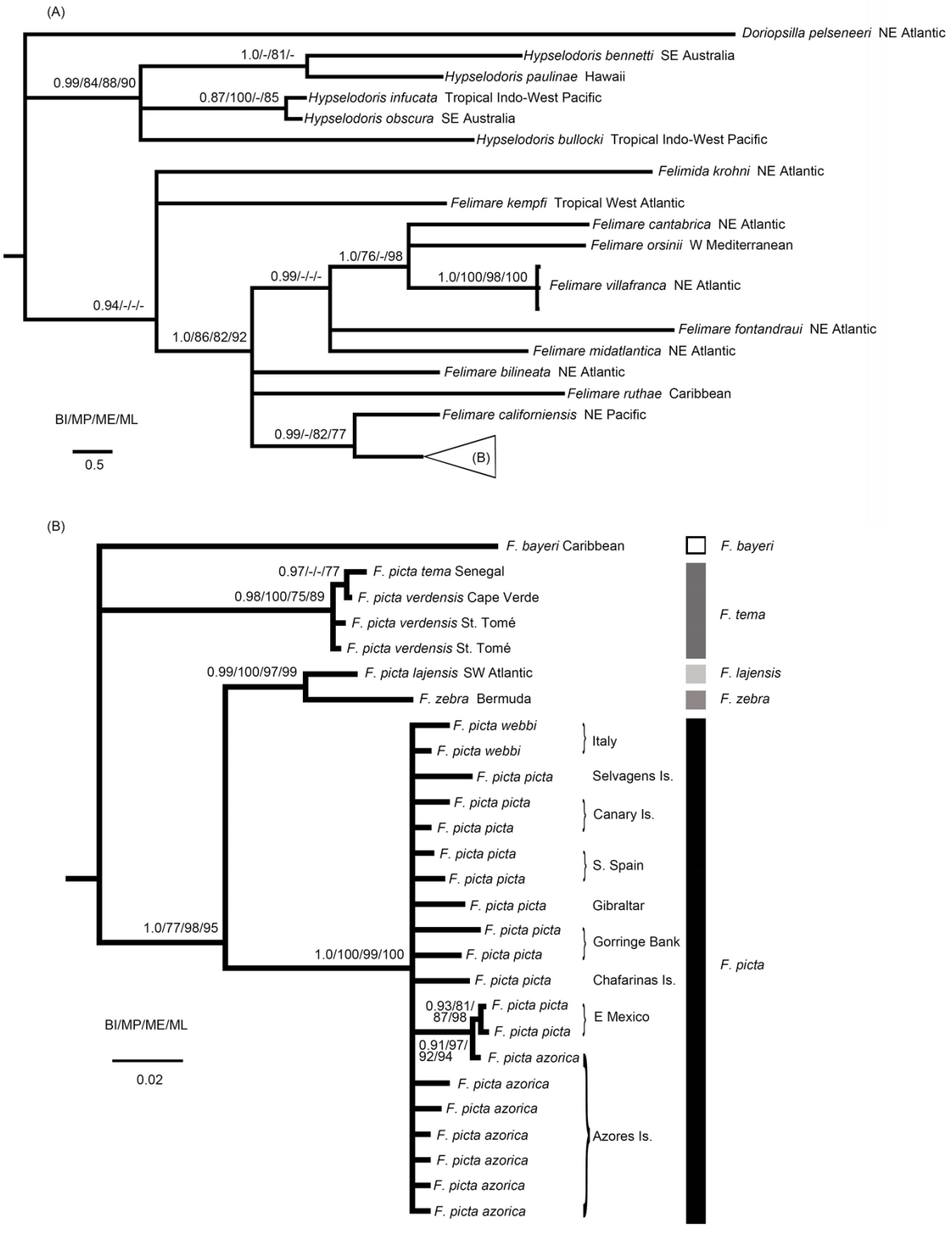
246 The results presented in Figure 1A support the distinctiveness of the eastern Pacific, Atlantic and
247 Mediterranean *Felimare* species compared to the Indo-Pacific *Hypselodoris* species. The
248 Caribbean *Felimare kempfi* failed to be included in a distinct clade with the remaining species of
249 this genus.

250

251 The *Felimare picta* complex

252 The monophyly of *Felimare picta*, including specimens from all geographical areas, is not
253 supported given the internal position of several west Atlantic species, such as *F. zebra* and *F.*
254 *lajensis* (Figure 1B). Although ME and BI recovered a third west Atlantic species, *F. bayeri*, as
255 the sister species of the African subspecies of *F. picta* (*F. p. tema* and *F. p. verdensis*), this
256 phylogenetic relationship was not supported by ML or MP methods. As a precautionary measure
257 these incongruent results were interpreted as an unresolved trichotomy (Figure 1B).
258 Nevertheless, *F. bayeri* was always recovered in a clade, including the remaining taxa of the *F.*
259 *picta* complex, with very high support values. These results alone show that *F. picta*, as currently
260 defined, constitutes a paraphyletic group.

261



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263

Figure 1 – (A) Phylogenetic relationships between several Indo-West Pacific *Hypselodoris* species and eastern Pacific, Atlantic and Mediterranean *Felimare* species obtained from the 16S+COI molecular data. Bayesian posterior probabilities (BI) and MP, ME and ML bootstrap support are shown above and below each node, respectively. (B) Phylogenetic relationships of the Atlanto-Mediterranean "*Felimare picta* complex"

The results presented in Figure 1B also confirm that, unlike other species of the same genus, the distribution range of *F. picta* encompasses both margins of the Atlantic Ocean together with several North Atlantic islands, namely the Azores. Interestingly, samples from this central Atlantic archipelago had identical or similar haplotypes compared to others from Mexico (west Atlantic), Portugal and Spain (east Atlantic) or Italy (Mediterranean).

The paraphyly of *F. picta* and, consequently, the phylogenetic relationships between its subspecies and other related taxa requires a revision of the taxonomic status of this complex of species and raises the question of how many transatlantic colonisation events could have occurred within the *F. picta* complex alone.

4. Discussion

4.1. Taxonomic implications

Although presenting a distinct color pattern when compared to other *F. picta*, the genetic data presented here suggests that the Azorean samples are not isolated from the west or east Atlantic and Mediterraneam samples. Although these differences in phenotype may result from recent genetic divergence or distinct ecological influences , the classification of taxa into different species or subspecies should be accompanied by genetic studies that are able to demonstrate their monophyly. The results are compatible with two alternative hypotheses: (i) genetic isolation between *F. picta* subspecies was recent, requiring genetic markers that evolve more quickly than those used in the present study, such as microsatellites, to detect reproductive isolation or; (ii) at least some of these "subspecies" coexist and interbreed. Considering present results and the fact that Ortea, Valdés & Garcia-Gomez (1996) did not describe valid morphological characters or designate type specimens to justify the erection of several new subspecies (e.g. *F. p. azorica*), *F. p. picta*, *F. p. webbi* or *F. p. azorica* should, by order of precedence and for the time being, be

295 synonymized under *F. picta* (Schultz, 1836) instead of assigning them to different subspecies.
296 This is particularly important within the chromodorids, which are known to be quite diversified
297 in terms of color patterns. In fact, some of the characters described for some subspecies, such as
298 the absence of yellow lines in *F. p. azorica*, are also observed in *F. p. picta* and the lack of
299 discriminating differences is even more evident between the latter and *F. p. webbi*.
300 *F. p. tema* (Edmunds, 1981) and *F. p. verdensis* (Ortea, Valdés & García-Gómez, 1996) show no
301 genetic differences between samples collected from Senegal and from Cape Verde. These results
302 are not surprising since Ortea, Valdés & García-Gómez (1996) reclassified *F. tema*, originally
303 described by Edmunds (1981) from Ghana as a subspecies of *F. picta*, and described a new
304 subspecies: *F. p. verdensis*, endemic from Cape Verde Islands, São Tomé and southern Angola.
305 However, they did not designate any type specimens and the morphological variability described
306 by Edmunds (1981) for *F. tema* is similar to that described for the specimens of *F. p. tema* and *F.*
307 *p. verdensis* except for one difference related to radula secondary denticles which are absent in
308 *F. p. tema*. These specimens are characterized by longitudinal orange lines, orange submarginal
309 border, orange lined gills and a dark blue background color with lighter blue patches along the
310 submarginal mantle border.
311 The results presented here suggest that, for the time being, the original taxonomic classification
312 by Edmunds (1981) should be reinstated and *F. tema* should include both *F. p. tema* and *F. p.*
313 *verdensis*. In fact, this tropical eastern Atlantic species, whose distribution area would now
314 include Senegal, Ghana, south Angola and the archipelagoes of Cape Verde and São Tomé, is
315 more distantly related to other parapatric norheastern Atlantic *F. picta* than the allopatric western
316 Atlantic *F. lajensis*.
317
318 *F. p. lajensis* Troncoso, Garcia & Urgorri, 1998 was originally described for Brazil as a
319 subspecies of *F. picta* but has been re-assessed as a different species by Dominguéz et al. (2006).
320 Its ambiguos taxonomic status (species or subspecies) is rooted on the morphological
321 descriptions that: i) distinguish this taxon from remaining *F. picta*, based on the dark blue to
322 violet body pattern with blue to violet gills, five spaced dorsal lines and a deferent duct with a
323 narrow preprostatic portion (Troncoso, García & Urgorri, 1998 and Domínguez, García &
324 Troncoso, 2006) or ii) suggest that it should be included in *F. picta* because some specimens
325 have gill rachis with yellow lines, up to nine dorsal yellow lines and a yellow or white mantle

margin (DaCosta, Padula & Schrödl, 2010). In fact, *F. picta* present gill rachis with yellow lines, only three dorsal lines and the preprostatic portion of the deferent duct is absent. DaCosta, Padula & Schrödl (2010) also showed that radular and allosperm receptacles differences described by Troncoso, García & Urgorri (1998) and Domínguez, García & Troncoso (2006) were not supported by the additional material analysed by those authors. Due to the wide morphological variation DaCosta, Padula & Schrödl (2010) decided to keep this taxon as a subspecies of *F. picta* until detailed anatomical comparisons and also molecular approaches are concluded to better understand not only the satus of this taxon but also the relationships between all *F. picta* subspecies.

In our study *F. picta* samples from Brazil were always recovered in a clade with *F. zebra* (Bermuda) and the genetic distance suggests a close relationship between these taxa. This southwestern Atlantic taxon is the sister clade to all remaining *F. picta*, excluding the east tropical Atlantic *F. tema* which diverged near the base of the *F. picta* complex as did also *F. bayeri*.

Although our results using two mitochondrial markers are clear and unambiguous, phylogenies based on mitochondrial DNA may result in misleading speciation histories when there are discrepancies with nuclear DNA (e.g. Zhang and Hewitt, 1996; Shaw, 2002). Joint analysis of mitochondrial and nuclear DNA may be informative even when results from both types of fragments are contradictory, particularly ”when the context for the conflict is understood” (Rubinoff and Holland, 2005). In fact, nuclear phylogenies frequently reinforce mitochondrial phylogenies (e.g. Levy et al. 2011). On the other hand, nuclear loci present several technical limitations in phylogenetic inference such as low copy number, heterozygosity, paralogous loci with multiple copies and low substitution rate, which make them uninformative when comparing close related species or even subspecies (for a revision see Rubinoff & Holland, 2005). One example of this argument are the chromodorid species analysed by Ortigosa et al. (2014) where nuclear markers did not add any additional information or resolve ambiguous results on closely related *Felimida* species. Although, reclassifying *F. picta*, *F. tema* and *F. lajensis* as independent species should be considered provisional until more specimens are throughly analysed and nuclear markers are compared, the suggestions presented above clarify the taxonomy and help to

356 define groups that are monophyletic and well characterized with mitochondrial DNA markers
357 and morphological characters.
358
359 In a wider phylogenetic scope the results indicate a basal politomy with four branches. Western
360 Atlantic *F. ruthae* and eastern Atlantic/west Mediterranean *F. bilineata* appear as individualized
361 branches. A third clade includes the *F. picta* complex, with the amphi-Atlantic *F. picta*, western
362 Atlantic *F. lajensis*, *F. zebra* and *F. bayeri* and eastern Atlantic *F. tema* and its sister species, the
363 eastern Pacific *F. californiensis*. Future phylogenetic studies will show if other eastern Pacific
364 species are included in the same or in different clades together with the remaining Atlantic and
365 Mediterranean species. A forth clade includes the remaining eastern Atlantic and Mediterranean
366 species: *F. cantabrica*, *F. fontandraui*, *F. orsinii*, *F. midatlantica* and *F. villafranca*. The
367 exclusion of *F. kempfi* from the *Felimare* clade was already argued by Johnson & Gosliner
368 (2012) placing this Caribbean species with the eastern Pacific *F. porterae* as a potential sister
369 group to a larger clade of eastern Pacific, Atlantic and Mediterranean *Felimare* species (Johnson
370 & Gosliner 2012).
371 The fact that the Indo-Pacific species are included in a distinct monophyletic clade corroborates
372 the conclusions of Johnson & Gosliner (2012) pointing to an ancient diversification of the
373 *Felimare* species in the eastern Pacific, Atlantic and Mediterranean since the closure of the
374 Tethys Sea in the East by the end of the Miocene.
375
376 *4.2. Felimare picta complex biogeography*
377 Sea surface currents and larvae characteristics, including larval behaviour, are crucial for an
378 organism to be able to cross important biogeographic barriers. Briggs (1995) described four
379 biogeographic provinces that are relevant to this study: northeastern Atlantic Lusitania (including
380 Macaronesia and the Mediterranean) (but see Almada et al., 2013), tropical West Africa, the
381 Caribbean and Brazil. These provinces are delimited by several soft (non-terrestrial) barriers
382 such as: (1) the mid-Atlantic barrier; (2) the Mauritanian cold water barrier in the northwestern
383 coast of Africa; (3) the Orinoco/Amazonas freshwater barrier; (4) the Almeria/Oran barrier
384 separating the Atlantic from the Mediterranean. These barriers impose restrictions to dispersal
385 but at the same time allow occasional crossings that result in the establishment of new
386 populations or species (e.g. Floeter et al., 2008).

Based on the phylogenetic results presented here the success of the *Felimare picta* complex crossing all these biogeographic barriers underlined by the amphi-Atlantic distribution of *F. picta* has no paralell among the chomodorids.

Panama Isthmus

The higher species richness in the west Atlantic is probably related with the close relationship between many species found in the Caribbean Sea and the ones found in the eastern Pacific, with several sister species being present on both sides of the Isthmus of Panama (Lessios, 2008). Examples of these sister relationships across this particular vicariant event include opisthobranchs (e.g. Malaquias & Reid, 2009) and also fish (Briggs, 1995; Muss et al., 2001). This pattern is also observed between the eastern Pacific *F. californiensis* and the *F. picta* complex. In fact, the *F. picta* complex is more closely related with *F. californiensis* than with other Atlanto-Mediterranean *Felimare* species which probably means that the origin of this species complex is posterior to the closure of this hard (terrestrial) biogeoraphic barrier.

Mid-Atlantic Barrier

The Atlantic ocean barrier is an important constraint to the migration of individuals between both sides of the Atlantic (Briggs, 1995). However this barrier has been crossed several times by many species that presently show an amphiatlantic distribution or that have sister taxa on both margins of the Atlantic reflecting historical speciation events (Carmona et al., 2011).

The genus Felimare and, in particular, the *Felimare picta* complex, illustrates both these patterns. Present geographic distribution of *F. picta* includes the west and eastern Atlantic Ocean and also the Mediterranean Sea which is supported by molecular data presented here. Although this hypothesis should be tested in the future with additional samples and appropriate phylogeographic analysis, the phylogeny of this group suggests that the mid-Atlantic barrier was probably crossed twice by this complex of species. First, an earlier colonization event resulted in the divergence of *F. tema* (including *F. picta tema* and *F. picta verdensis*) in equatorial Africa region. Second, a separation between the clade *F. lajensis*/*F. zebra* and *F. picta* (including *F. picta* from western and northeastern Atlantic) and a transatlantic migration resulting in the colonization of both sides of the Atlantic by this later species.

Major oceanic surface currents suggest that this migration could have followed a westward route, particularly in the equatorial region. With predominant surface currents from northwest Africa to Central America (North equatorial current) and from southwest Africa to southeast South America (South equatorial current), one hypothesis to explain current species distribution would be an westward migration with speciation along the American coast. However this westward migration hypothesis is highly improbable if we consider the phylogeny of this group of species, particularly the basal position of the west Atlantic species within the *F. picta* complex phylogeny and the eastern Pacific *F. californiensis* being the sister species of this clade.

An alternative hypothesis would be an eastward colonization from the western Atlantic to the European and African coasts which, if we assume that the present surface current pattern was already in effect, could follow two alternative routes: a northern route along the Gulf stream followed by the Azores and the Canaries currents (Barton, 2001) and an equatorial route following the north equatorial countercurrent (see Fonseca et al., 2004). Phylogenetic patterns showed in this work and those reported by other authors based on morphological and meristic data (Gosliner & Johnson, 1999 and Alejandrino & Valdés 2006) also support this eastward migration hypothesis.

Assuming that the origin of *F. picta* is posterior to the closure of the Isthmus of Panama and therefore posterior to the settlement of the Gulf Stream, the similarity between Mexican samples of *F. picta* and an individual collected in the Azores suggest that this archipelago may have acted, and still acts, as a stepping-stone in this northern route. This hypothesis is further supported by the fact that during glacial periods these currents were even stronger than in present times (Wunsch, 2003) which could result in the rapid transportation from west to the east Atlantic of planktotrophic larvae or adults and eggs on rafting materials. The equatorial route is seasonal, being stronger during the Spring (Richardson et al., 1992), when it reaches surface transport velocities between 23 cm s^{-1} (Richardson et al., 1992) and 45 cm s^{-1} (Urbano, Almeida & Nobre, 2008). This would mean that the mid Atlantic barrier could be crossed at maximum velocity in less than 10 days (38.88 Km day^{-1}). This would allow an independent eastward migration by a southern route which could have led to the origin of *F. tema* in the tropical west African coast. Only future phylogeographic studies with population samples of *F. picta* from both sides of the Atlantic may shed some light on the dispersion route of this species.

448 Mauritanian cold water barrier

449 The present allopatric distribution of *F. tema* and *F. picta* could be explained by the persistence
450 of biogeographic barriers and/or by ecological constraints. The cold water barrier along the
451 Mauritanian shores due to strong upwelling (Marañón et al., 2001) and the Pleistocenic
452 glaciations could have prevented *F. tema* from colonizing the northeastern Atlantic shores. More
453 recently, with the settling of *F. picta* in the northeastern Atlantic shores other ecological
454 constraints may have been in effect. This argument is based on the fact that *F. tema* is deeply
455 rooted within a clade with several west Atlantic extant species and *F. picta* shares a common
456 ancestor with *F. lajensis* and *F. zebra*, which are also west Atlantic species. If the hypothesis of
457 the "eastward migration" proves to be correct, present allopatric distribution of *F. tema* and *F.*
458 *picta* may be the result of two independent dispersion processes: the first resulting in the
459 speciation of the tropical east Atlantic *F. tema* from an west Atlantic ancestor, and the second
460 resulting in the colonization of the northeastern Atlantic and the Mediterranean by *F. picta*.
461 Examples of this eastward migration are common across a large array of taxonomic groups and
462 are much more common than migrations on the opposite directions (Ávila, 2005; Rocha et al.,
463 2008; Beldade et al., 2009). This may be the result of the predominant current patterns described
464 above and the higher species richness in the west Atlantic shores.
465
466 Orinoco/Amazonas Barrier
467 Genetic isolation between central western and southwestern Atlantic may have occurred about 6
468 million years with the origin of the Amazon River (Nunan, 1992; Hoorn 1994). Variation in
469 salinity levels and the absence of appropriate hard substrate across the Central American Gap
470 could also increase this isolation effect (Rocha, 2003; Ludt & Rocha 2014). Since the closure of
471 the Isthmus of Panamá the patterns of epipelagic circulation remained approximately constant
472 (Haug & Tiedemann, 1998) therefore, conditions were appropriate for this biogeographic semi-
473 permeable barrier to promote a recent split between *F. lajensis* and *F. zebra*.
474
475 A question remains: among all chromodorids why has only the *F. picta* complex experienced
476 such a success crossing all main Atlantic biogeographic barriers? Even considering *F. picta*
477 sensu strictu, this is the only chromodorid whose distribution encompasses northwestern
478 Atlantic, northeastern Atlantic and the Mediterranean. Futhermore, nudibranchs and the

chromodorids, in particular, are sedentary and have a dispersion ability that is much reduced during all life stages. Rafting on floating materials could explain their ability to colonize distant locations, however the individuals of these species are benthic and are usually found over sponges upon which they feed. Therefore it is improbable that these organisms may disperse during their adult phase. Coelho & Calado (2010) repported that *F. picta* shows the largest egg size and planktotrophic larvae length at hatching reported among nudibranch molluscs (for a review see Todd, Lambert & Davies, 2001). Although *F. villafranca* have even larger eggs, it presents direct and not planktotrophic development. It is commonly accepted that large larvae have higher survival rates during transport in the water column. Although, Shulman & Bermingham (1995) found no relationship between oceanographic patterns, larval duration and population genetic structuring in the Caribbean, they refer that a different scenario could emerge on larger geographic scales. For this purpose, additional studies on different species which were also able to cross the MAB, such as the Chromodorididae *Tyrinna evelinae* and also *Cadlina rumia*, from a sister group of the Chromodorididae (Johnson, 2011), could be of extreme interest. From an ecological point of view, assignment tests of recruits could indicate if the most likely sources of the recruits were local or distant populations (see Piry et al., 2004; Wilson & Rannala, 2003).

A comprehensive study of the phylogeny of the genus Felimare, including all its species and nuclear DNA markers is still needed to clarify the taxonomy of this group. Furthermore, phylogeographic data would provide information on the direction and number of colonization events of each taxonomic entity and would allow the implementation of species delimitation analysis (see Puillandre et al., 2012).

Nevertheless the biogeographic considerations and phylogenetic relationships described above may help to refine current information on a group of marine organisms that have been raising the attention of a broad community from evolutionary biology and ecology to natural products chemistry.

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Figure 1

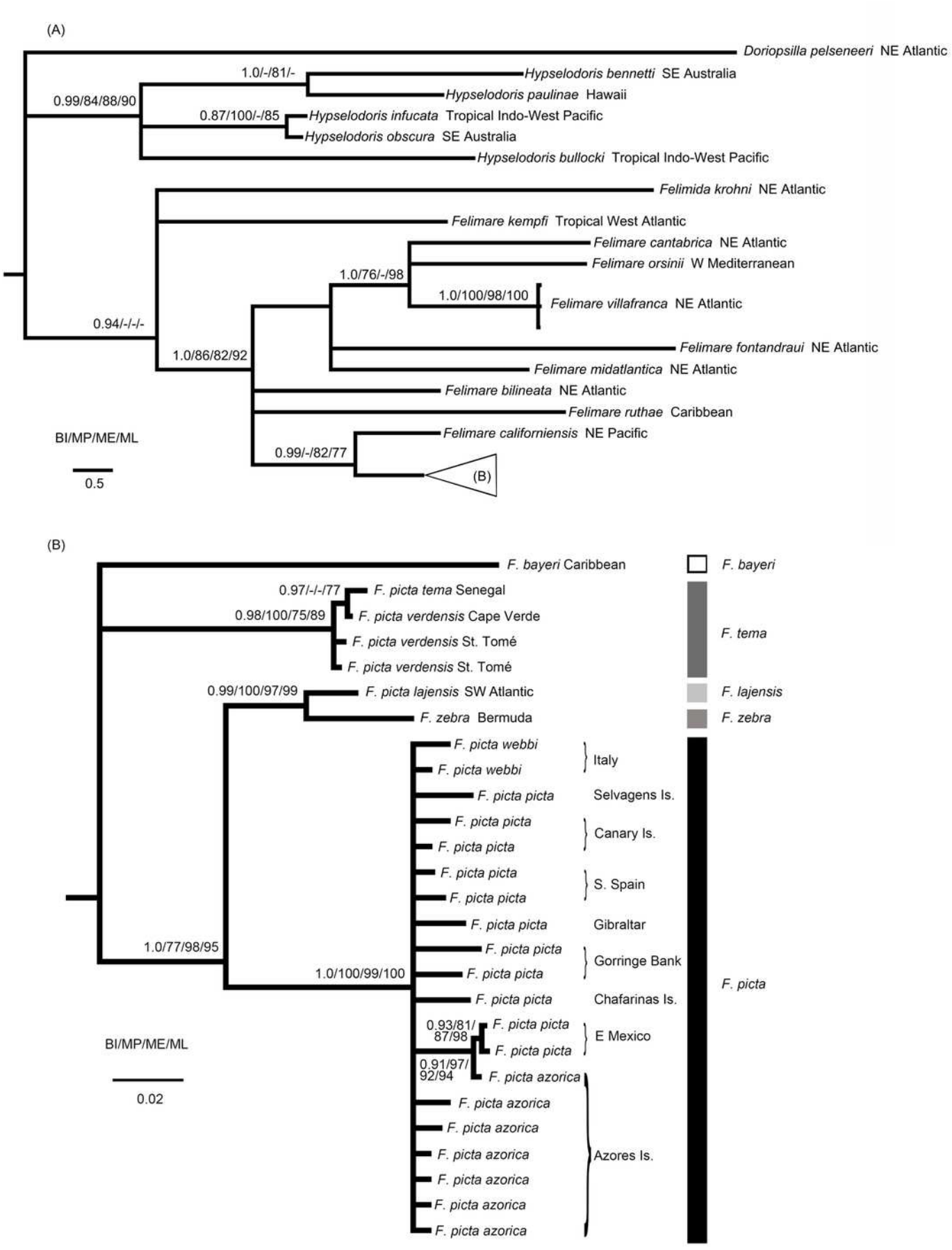

Table 1(on next page)

Estimates of net evolutionary divergence

Table 1 – Estimates of net evolutionary divergence between groups of sequences from *F. picta* subspecies, other Atlanto-Mediterranean *Felimare* species and Indo-West Pacific *Hypselodoris* species. The number of base substitutions per site from estimation of net average between groups of sequences are shown. Standard error estimate(s) are shown above the diagonal. The *Felimare picta* complex is highlighted in bold. Analyses were conducted using the Maximum Composite Likelihood model (Tamura, Nei & Kumar, 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.426). The analysis involved 45 mtDNA sequences and all ambiguous positions were removed resulting in a total of 1124 nucleotides in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

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	Chromodoris	*F. bayeri*	*F. bilineata*	*F. californensis*	*F. cantabrica*	*F. fontadraui*	*F. kempfi*	*F. lajensis*	*F. midatlantica*	*F. orsinii*	***F. picta azorica***	***F. picta picta***	***F. picta webbi***	***F. picta tema***	***F. picta verdensis***	*F. ruthae*	*F. villafranca*	*F. zebra*	*Hypsel*
Chromodoris		0,546	0,617	0,613	0,735	0,631	0,496	0,628	0,560	0,624	**0,690**	**0,702**	**0,691**	**0,565**	**0,560**	0,872	0,576	0,591	0,6
F. bayeri	1,766		0,284	0,218	0,470	0,395	0,489	0,079	0,307	0,389	**0,082**	**0,084**	**0,083**	**0,067**	**0,063**	0,582	0,370	0,082	0,6
F. bilineata	2,055	0,922		0,250	0,464	0,379	0,561	0,288	0,286	0,371	**0,289**	**0,290**	**0,273**	**0,278**	**0,269**	0,502	0,420	0,300	0,6
F. californensis	2,069	0,692	0,771		0,442	0,363	0,648	0,199	0,330	0,382	**0,197**	**0,197**	**0,192**	**0,182**	**0,179**	0,630	0,363	0,209	0,7
F. cantabrica	2,592	1,536	1,520	1,454		0,478	0,786	0,449	0,461	0,405	**0,422**	**0,432**	**0,427**	**0,444**	**0,429**	0,747	0,280	0,494	0,9
F. fontadraui	2,095	1,260	1,227	1,162	1,596		0,633	0,453	0,309	0,329	**0,481**	**0,479**	**0,480**	**0,395**	**0,381**	0,602	0,399	0,441	0,7
F. kempfi	1,660	1,611	1,865	2,211	2,752	2,217		0,519	0,586	0,653	**0,576**	**0,577**	**0,572**	**0,533**	**0,522**	0,845	0,679	0,542	0,7
F. lajensis	2,138	0,258	0,930	0,644	1,485	1,499	1,749		0,338	0,458	**0,050**	**0,050**	**0,050**	**0,074**	**0,074**	0,652	0,346	0,022	0,7
F. midatlantica	1,839	0,941	0,919	1,053	1,497	0,968	1,973	1,085		0,356	**0,362**	**0,365**	**0,370**	**0,316**	**0,320**	0,614	0,270	0,373	0,6
F. orsinii	2,044	1,194	1,175	1,206	1,373	1,059	2,208	1,489	1,147		**0,487**	**0,490**	**0,484**	**0,381**	**0,386**	0,627	0,206	0,440	0,6
F. picta azorica	**2,340**	**0,272**	**0,944**	**0,627**	**1,364**	**1,572**	**1,966**	**0,163**	**1,130**	**1,579**		**0,000**	**0,000**	**0,081**	**0,078**	**0,651**	**0,408**	**0,054**	**0,7**
F. picta picta	**2,379**	**0,277**	**0,949**	**0,628**	**1,405**	**1,564**	**1,973**	**0,165**	**1,138**	**1,591**	**0,000**		**0,000**	**0,083**	**0,080**	**0,647**	**0,412**	**0,053**	**0,7**
F. picta webbi	**2,338**	**0,272**	**0,891**	**0,617**	**1,387**	**1,569**	**1,957**	**0,164**	**1,155**	**1,569**	**0,000**	**0,000**		**0,084**	**0,080**	**0,636**	**0,404**	**0,055**	**0,7**
F. picta tema	**1,847**	**0,211**	**0,885**	**0,572**	**1,450**	**1,259**	**1,798**	**0,246**	**0,994**	**1,180**	**0,262**	**0,269**	**0,272**		**0,004**	**0,614**	**0,404**	**0,089**	**0,6**
F. picta verdensis	**1,826**	**0,200**	**0,866**	**0,570**	**1,406**	**1,207**	**1,767**	**0,250**	**1,019**	**1,218**	**0,255**	**0,259**	**0,260**	**0,008**		**0,600**	**0,397**	**0,090**	**0,6**
F. ruthae	3,040	1,936	1,691	2,161	2,538	2,027	2,945	2,218	2,051	2,156	**2,212**	**2,206**	**2,161**	**2,090**	**2,035**		0,620	0,614	0,8
F. villafranca	1,920	1,128	1,313	1,124	0,901	1,274	2,339	1,068	0,830	0,635	**1,269**	**1,283**	**1,256**	**1,263**	**1,247**	2,112		0,362	0,6
F. zebra	1,942	0,258	0,943	0,650	1,617	1,441	1,841	0,072	1,192	1,388	**0,181**	**0,179**	**0,185**	**0,281**	**0,287**	2,045	1,100		0,7
Hypselodoris	2,209	2,135	2,113	2,736	3,266	2,459	2,473	2,693	2,350	2,401	**2,585**	**2,577**	**2,484**	**2,423**	**2,424**	2,978	2,182	2,463	

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