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ORIGINAL ARTICLE

First molecular phylogeny of the subfamily Polycerinae (Mollusca, Nudibranchia, Polyceridae)

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Abstract The subfamily Polycerinae includes four genera with around 46 species described to date. This subfamily is characterized by a limaciform body, which may have simple tentacular processes on the margin of the oral veil. Phylogenetic relationships between the genera of the subfamily Polycerinae (Polyceridae) have not yet been studied, and therefore, the only available information is based on morphological descriptions. The present study reports the first phylogenetic analysis of Polycerinae based on the mitochondrial genes cytochrome oxidase subunit I and the large ribosomal subunit (16S rRNA) using maximum likelihood and Bayesian methods. Our results showed that Polycerinae is monophyletic, but the relationships within the subfamily as well as within *Polycera* remain unresolved. A key finding of this study is that there are clearly two sympatric species of *Polycera* present in South Africa: *Polycera capensis* Quoy and Gaimard, 1824 also found in Australia and an undescribed *Polycera* sp. On the other hand, the studied specimens of the genus *Gymnodoris* were clustered within Polycerinae, reopening the problem of the systematic position of this genus. Additional genes and species of Polycerinae and *Gymnodoris* would provide more information and probably fully resolve this situation.

Keywords COI · 16S · *Gymnodoris* · Nudibranchia · Polycerinae · *Polycera capensis*

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Introduction

The absence of nudibranchs in fossil records has complicated the understanding of their biology and evolution (Valdés 2001). Despite this limitation, many studies based on classical morphology have been undertaken since the nineteenth century (e.g., Alder and Hancock 1845–1855; Bergh 1877, 1890, 1902, 1906; Odhner 1934; Valdés and Gosliner 1999; Wägele and Willan 2000; Valdés 2001, 2002; Fahey and Gosliner 2001, 2004; Gosliner 2004; Pola et al. 2005a, 2006a). However, a taxonomy based only on morphology has its limits (Medina and Walsh 2000; Wiens and Penkrot 2002; Fall et al. 2003; Dayrat 2005). Morphological characters may vary within the same species as the result of selective pressures and adaptation to varying environmental parameters (Wägele 2005), and the choice of taxonomically informative characteristics depends on the criteria of taxonomists (Mikkelsen 1998). Molecular techniques can palliate these limitations (Medina and Collins 2003; Wägele et al. 2003; Wägele 2005; Ornelas-Gatdula et al. 2012; Pola et al. 2012; Carmona et al. 2013). Mitochondrial genes have provided interesting information on the phylogeny of nudibranchs (Thöllesson 1999a, b, 2000; Medina et al. 2001; Wollscheid-Lengeling et al. 2001; Fahey 2003; Valdés 2003; Wilson and Lee 2005; Pola et al. 2007; Turner and Wilson 2008; Johnson 2010; Pola and Gosliner 2010; Johnson and Gosliner 2012; Carmona et al. 2013). The results of these researches have

clarified the relationships within some groups, but there are still many unclear phylogenetic relationships, for example, within species grouped under the family Polyceridae Alder and Hancock, 1845 (superfamily Polyceroidea, subclade Doridacea, clade Euctenidiacea; Bouchet and Rocroi 2005). This family includes a group of nudibranchs that have elongate and limaciform bodies and a reduced mantle skirt. Their lamellate rhinophores have a pocket, and sometimes a sheath into which they can retract, although their gills are non-retractile. In the past, it was related to notodorids and gymnodorids. Old classifications included gymnodorids as subfamily Gymnodoridinae within the family Polyceridae (Eliot, 1903), although other authors such as Odhner (1941) believed they should be regarded as distinct. Macnae (1958) considered that gymnodorids and polycerids shared enough characters to belong to the same family. Both have their principal ganglia concentrated into a compact mass above and alongside the esophagus. Their pleural and cerebral ganglia are usually enclosed in the same sheath and are not easily distinguishable when viewed from above. Their abdominal ganglion is drawn up so as to lie close to the pleural ganglion on the right side. They have a blood gland in association with the anterior aorta, lying in the mass of connective tissue overlying the posterior portion of the anterior genital mass. Only the buccal region and its armature, the form of the radula and its teeth, are different in both families and are used as diagnostic characters even at species level (Macnae 1958). Nowadays, Gymnodorididae is considered a separated family (Bouchet and Rocroi 2005; Bouchet 2013) although, to date, the relationship between gymnodorids and polycerids remains unresolved.

At present, Polyceridae comprises four subfamilies: Polycerinae, Triophinae, Nembrothinae and Kalinginae (Burn 1967; Rudman 1998). Ortea et al. (2004) proposed a new subfamily, Kankelibranchinae, based on a unique specimen from Cuba. In the subfamily Triophinae, the genera *Kaloplocamus* and *Plocamopherus* have been revised (Vallés and Gosliner 2006). However, information about the genera *Crimora*, *Limacia* and *Triopha,* among others, is still very limited. Nembrothinae, with its genera *Tambja*, *Nembrotha* and *Roboastra,* have been quite intensely studied (Pola et al. 2003, 2005a, b, c, 2006a, b, c, 2007, 2008a, b). The remaining subfamilies still remain genetically unexplored.

The subfamily Polycerinae is characterized by a limaciform body, which may have simple tentacular processes on the margin of the oral veil. On the sides of the body, the mantle is reduced to a few tentacular papillae, which are sometimes connected by a ridge. Rhinophores are lamellate, while and gills are often simple pinnate. They lack buccal pump and have large chitinous jaws usually with a wing-like accessory process. The two innermost lateral teeth on each side are usually large, bicuspidate and hooked, while the outer teeth are much smaller and acuspidate plates. The penis is usually armed with spines (Rudman 1998; Wägele and Willan 2000). There are four genera included within Polycerinae: *Polycera* Cuvier, 1817 (31 species), *Polycerella* Verril, 1880 (2 species), *Palio* Gray, 1857 (6 species) and *Thecacera* Fleming, 1828 (7 species), with around 46 species described plus two considered as *nomina dubia*, *Polycera funerea* Pruvot-Fol, 1930 and *Polycera pruvotae* Risbec, 1953 (Boxshall et al. 2013). Phylogenetic relationships within Polycerinae and between Polyceridae subfamilies are unknown. There are only some monographs referring to some Polycerinae species (Müller 1776; Quoy and Gaimard 1824; MacFarland 1905; Lemche 1929; Marcus 1964; Robilliard 1971; García and Bobo 1984), and few taxa have been included in phylogenetic studies focused on other families or with other aims (Wollscheid and Wägele 1999; Thöllesson 1999b, 2000; Pola et al. 2007; Pola and Gosliner 2010).

Within *Polycera*, the taxonomic status of *Polycera capensis* Quoy and Gaimard, 1824 is repeatedly a matter of debate. It was originally described from South Africa and lately commonly reported from Sydney to Port Stephens in New South Wales and eastern Australia (Rudman 1998). It is thought that *P. capensis* was introduced from South Africa to Australia by fouling, and it has spread 200 km since then (Macnae 1958; Willan 2000) although some authors have considered the Australian *Polycera capensis* to be a different species (Allan 1932; Thompson 1975). Taking into account the variability shown in this subfamily by other species, for example *Polycera quadrilineata* (Alder 1841; Alder and Hancock 1845; Odhner 1941), a more complete phylogenetic study of *Polycera capensis* is needed to shed light on this controversial matter.

The objectives of this study are to: (1) test the monophyly of Polycerinae giving the first molecular insight of the phylogenetic relationships within this subfamily, with emphasis on the genus *Polycera*; (2) discuss the relationship between polycerids and gymnodorids; and (3) evaluate the conspecificity of specimens of *Polycera capensis* from South Africa and Australia.

Materials and methods

Samples analyzed

Samples were obtained from specimens deposited at the Museo Nacional de Ciencias Naturales, Madrid (MNCN), the California Academy of Sciences, San Francisco (CASIZ), and the Museu Municipal de Funchal, Madeira (MMF). DNA was successfully extracted from 50 samples, corresponding to 37 specimens and 13 species of the

Table 1 Specimens used for molecular analysis, locality, voucher and GenBank accession number

Species	Locality	Voucher	GenBank Accesion Number	
			COI	16S
Polycera quadrilineata	United Kingdom, Oban	MNCN15.05/46738	EF142907	EF142953
Polycera quadrilineata	Kattegat, North Sea	GB1	–	AF249229
Polycera quadrilineata	Sweden, Bohuslän, Kristineberg	GB2	AJ223275	AJ225200
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55458	–	JX274040
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55462	JX274070	JX274041
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55465	JX274071	JX274042
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55456	JX274072	JX274043
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55466	JX274073	JX274044
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55463	JX274074	JX274045
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55464	JX274075	–
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55457	JX274076	–
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55459	JX274077	JX274046
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55460	JX274078	–
Polycera quadrilineata	Sweden, Gothenborg, Tjärnö	MNCN15.05/55455	JX274079	JX274047
Polycera faeroensis	Portugal, Estacada, Aveiro	MNCN15.05/55503.1	JX274088	–
Polycera faeroensis	Portugal, Estacada, Aveiro	MNCN15.05/55503.2	JX274089	JX274056
Polycera aurantiomarginata	Spain, Cádiz	GB	AJ223274	AJ225199
Polycera aurantiomarginata	Morocco, Aghroud	MNCN15.05/55483	–	JX274037
Polycera aurantiomarginata	Morocco, Aghroud	MNCN15.05/55492	JX274068	JX274038
Polycera aurantiomarginata	Morocco, Aghroud	MNCN15.05/55490	JX274069	JX274039
Polycera hedgpethi	Morocco, Aghroud	MNCN15.05/55493	JX274086	–
Polycera atra	California, San Francisco Bay, San Francisco Marina	CASIZ170506a	JX274084	JX274052
Polycera atra	California, San Francisco Bay, San Francisco Marina	CASIZ170506b	JX274085	JX274053
Polycera capensis	South Africa, Western Cape province, Hout Bay	CASIZ176907	HM162687	HM162597
Polycera capensis	South Africa, Cape province, Atlantic coast, Oudekraal	CASIZ176280	JX274091	–
Polycera capensis	South Africa, Western Cape Province, False Bay	CASIZ176375	JX274092	JX274058
Polycera cf. *capensis*	Australia, New South Wales, Nelson Bay	MNCN15.05/55470	JX274083	JX274051
Polycera tricolor	California, San Francisco Estuary, San Francisco Bay, Marin County	CASIZ76438a	JX274087	JX274054
Polycera tricolor	California, San Francisco Estuary, San Francisco Bay, Marin County	CASIZ76438b	–	JX274055
Polycera sp. A	South Africa, Western Cape Province, False Bay, Gordon's Bay	CASIZ176169	JX274081	JX274049
Polycera sp. A	South Africa, Eastern Cape Province, Tsitsikamma Coastal National Park	CASIZ176387	JX274082	JX274050
Polycera sp. B	Hawaii, Maui, Maalaea Bay	CASIZ176795	JX274093	–
Polycera sp. C	Pacific Ocean, Marshall Islands, Kwajalein Atoll, Onemak Pinnacle	CASIZ120773	JX274090	JX274057
Polycerella emertoni	Spain, Cádiz	GB	AJ223273	AJ225198
Polycerella emertoni	Spain, Cádiz, Santi Petri	MNCN15.05/55480	JX274095	JX274060
Polycerella emertoni	Spain, Cádiz, Santi Petri, dock	MNCN15.05/55479.1	JX274096	–
Polycerella emertoni	Spain, Cádiz, Santi Petri, dock	MNCN15.05/55479.2	JX274097	–
Polycerella emertoni	Spain, Cádiz, Santi Petri, dock	MNCN15.05/55482[a]	JX274098	JX274061
Polycerella emertoni	Spain, Cádiz, Santi Petri, dock	MNCN15.05/55482[a]	JX274099	JX274062
Thecacera pennigera	Spain, Cádiz	GB	AJ223277	AJ225202

Table 1 continued

Species	Locality	Voucher	GenBank Accesion Number	
			COI	16S
Thecacera pennigera	South Africa, Cape province, Atlantic coast, Oudekraal	CASIZ176285	JX274094	JX274059
Palio dubia	Sweden, Bohuslän, Kristineberg	GB	AJ223272	AJ225197
Palio dubia	Sweden, Gullmaren	MNCN15.05/55467	JX274100	–
Gymnodoris alba	Australia, New South Wales, Nelson Bay	MNCN15.05/55472	JX274101	JX274063
Gymnodoris striata	Australia, Queensland	GB	HQ987955.1	–
Plocamopherus maderae	Cape Verde Archipelago, Sal Island	MNCN15.05/46735	EF142905	EF142951
Plocamopherus tilesii	Australia, New South Wales, Nelson Bay	MNCN15.05/55475	JX274102	JX274064
Plocamopherus imperialis	Australia, New South Wales, Nelson Bay	MNCN15.05/55468	JX274103	JX274065
Kaloplocamus ramosus	Portugal, Azores	GB	EF142904	–
Kaloplocamus ramosus	Australia, New South Wales, Nelson Bay	MNCN15.05/55473	JX274104	JX274066
Kaloplocamus ramosus	Australia, New South Wales, Nelson Bay	MNCN15.05/55471	JX274105	–
Crimora lutea	Australia, Western Australia: Abrolhos Is.	MNCN15.05/46737	EF142903	EF142950
Crimora papillata	Spain, Mediterranean Sea	GB	AF249821	–
Triopha maculata	California, Marin County, Duxbury Reef	CASIZ181556	HM162691	HM162601
Triopha catalinae	California, San Francisco, San Francisco Yacht Harbor	CASIZ170648	HM162690	HM162600
Limacia clavigera	Spain, Cádiz, Bajo Cabezuela	MNCN 15.05/46736	EF142906	EF142952
Nembrotha mullineri	Philippines, Malapascua Is., Lapus–Lapus	MNCN 15.05/46723	EF142895	EF142944
Nembrotha guttata	Philippines, Siguijor Island, Siguijor Wall	WAMS11556	EF142894	EF142943
Roboastra luteolineata	Japan, Okinawa, Kerama Is., Zamami Is	MNCN15.05/46731	EF142861	EF142910
Roboastra caboverdensis	Cape Verde, Santo Antão Is	MNCN 15.05/46614	EF142859	EF142908
Tambja simplex	Cape Verde, Isla de San Vicente	MNCN 15.05/46680	EF142874	EF142925
Tambja fantasmalis	Cape Verde, Boavista Is., Baía das Gatas	MNCN 15.05/46734	EF142873	EF142924
Trapania hispalensis	Portugal, Aveiro	MNCN15.05/55504	JX274080	JX274048
Okenia rosacea	United States, California, Marin County, Duxbuty Reef	CASIZ 184340	KF192605	–
Okenia amoenula	South Africa, False Bay	CASIZ 176191	KF192606	–
Ancula gibbosa	Sweden, Bohuslän, Kristineberg	GB	AJ223255	AJ225179
Montereina concinna	Australia, Great Barrier	GB	AF249801	AF249228
Jorunna tomentosa	Sweden, Bohuslän, Kristineberg	GB	AJ223267	AJ225191
Platydoris argo	Ceuta, Strait of Gibraltar	GB	AY345037	AY345037
Felimida krohni	Murcia, SE Spain	GB	AY345036	AY345036
Felimare picta	Spain, Atlantic	GB	AF249787	AF249238
Phyllidia elegans	Tab Island, Papua New Guinea	GB	AJ223276	AJ225201
Doriopsilla areolata	Cádiz, Andalucia, Spain	GB	AJ223262	AJ225186
Bathydoris clavigera	Antarctica, South Shetland Islands, Elephant Island	CASIZ167553	JX274106	JX274067

GB, sequences obtained from Genbank

[a] The whole specimen was used for the extraction, this voucher belongs to a tube with another 10 specimens recollected the same date and place

subfamily Polycerinae (10 *Polycera*, 1 *Polycerella*, 1 *Thecacera*, 1 *Palio*). PCR amplification of cytochrome oxidase subunit I (COI) and 16S rRNA genes was obtained for 38 and 30 samples, respectively. Sequences were submitted to GenBank, and the accession numbers are given in Table 1 as well as locality of origin and the voucher reference. DNA extraction and PCR amplification were carried out at the University of Oviedo (Spain).

In addition, in order to test the monophyly of Polycerinae, available sequences from the GenBank were used for species belonging to Polycerinae, Nembrothinae and Triophinae, plus species belonging to six families within Doridacea (Bouchet and Rocroi 2005): Discodorididae (*Montereina concinna, Jorunna tomentosa*, and *Platydoris argo*), Chromodorididae (*Felimida krohni*, and *Felimare picta*), Goniodorididae (*Ancula gibbosa*), Gymnodorididae

(*Gymnodoris striata*), Phyllidiidae (*Phyllidia elegans*) and Dendrodorididae (*Doriopsilla areolata*) (Table 1). As in other phylogenetic studies involving this family, *Bathydoris clavigera* was chosen as the outgroup (Millen and Martinov 2005; Pola et al. 2005a, 2006b, 2007), based on its condition of sister taxon (Wägele 1989; Wägele and Willan 2000; Schrödl et al. 2001; Wollscheid-Lengeling et al. 2001; Valdés 2002).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the preserved tissue samples either from the foot or the mantle using the Chelex® resin (Bio-Rad Laboratories) according Estoup's et al. (1996) protocol. The tissue was introduced in an Eppendorf tube with 500 µl of Chelex resin (10 %) and 7.5 µl of Proteinase K (20 mg/ml). It was incubated at 55 °C for 90 min (vortex every 15 min), and then introduced in an oven at 100 °C for 20 min to inactivate the enzyme. The tube was then stored at 4 °C or frozen at −20 °C for long-time preservation.

Polymerase chain reaction (PCR) was employed to amplify fragments of the COI and 16S rRNA genes. We used the primers designed by Palumbi et al. (1991) for 16S rRNA: 16Sar-L (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3′), and by Folmer et al. (1994) for COI: LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2 198 (5′-TAAACTTCAGGG TGACCAAAAAATCA-3′).

The amplification reaction was performed in a total volume of 40 µl, including 8 µl of Promega (Madison, WI) buffer 1×, 4 µl of $MgCl_2$ (25 mM), 4 µl dNTPs (2.5 mM), 2 µl of each primer (20 µM), 4–10 µl of template DNA and 1 U of DNA Taq polymerase (Promega).

The PCR conditions for COI were the following: an initial denaturing step at 94 °C for 10 min, then 40 cycles of denaturing at 94 °C for 1 min, annealing at 46 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 8 min. PCR conditions for the 16S gene were similar, except it was run for 35 cycles and the annealing temperature was 48 °C.

PCR products were visualized in 2 % agarose gels with 3 µl of 10 mg/ml ethidium bromide. Stained bands were excised from the gel, and DNA was purified with Wizard® SV Gel and PCR clean-up System (Promega) prior to sequencing. Then, DNA quantification and pre-sequencing PCR (initial denaturing step at 96 °C for 1 min followed by 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min, and final extension at 60 °C for 10 min) were carried out. Products were precipitated using standard 2-propanol precipitation and re-suspended in formamide. Sequencing was performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 terminator system, at the Sequencing Unit of the University of Oviedo (Spain).

Sequence edition and phylogenetic analysis

Sequences were assembled and edited using BioEdit Sequence Alignment Editor Software 7.0.5.3 (Hall 1999) and Geneious Pro 4.5.4 (Drummond et al. 2009). Geneious and MAFFT (Katoh et al. 2009) were employed to align the sequences, using the default settings in both programs. The alignments were checked by eye using MacClade 4.08 (Maddison and Maddison 2005). Protein-coding sequences were translated into amino acids for alignment confirmation. Saturation was examined through the substitution saturation test (Xia et al. 2003) implemented in DAMBE 5.2.14 (Xia 2000). For the COI gene, the number of transitions (*s*) and transversions (*v*) was plotted against the evolution model distances with the same program. Saturation plots were examined separately for the first, second and third codon positions.

The most variable regions from the 16S rRNA alignment were removed using both the default settings and the standard options for stringent and less stringent selection in Gblocks (Talavera and Castresana 2007). Excluding "indel-rich" regions, the tree was in general poorly resolved with lower node support. Therefore, final analyses were performed including all bases. Sequences of COI and 16S were trimmed to 643 and 465 base pairs, respectively.

Individual gene analyses and a concatenated analysis were performed. To test for conflicting phylogenetic signal between genes, the incongruence length difference (ILD) test (Farris et al. 1995) was conducted as the partition homogeneity test in PAUP 4.0b10 (Swofford 2003). Test settings consisted of 10 random stepwise additions (100 replicates) with TBR branch swapping.

The software jModelTest 0.1 (Posada 2008) was employed to determine the best-fit nucleotide substitution model for each gene and accompanying evolutionary parameter values for the data. ProtTest 1.4 (Abascal et al. 2005) was employed to infer the best-fit model of protein evolution. Akaike information criterion (AIC) (Akaike 1974) was used in both programmes to find the best evolutionary model. The evolution model obtained for the 16S rDNA was the Transitional model +I+G (Rodriguez et al. 1990), with a gamma shape value of 0.3670. The General Time Reversible +I+G (Tavaré 1986) was the evolution model for COI gene and concatenated sequences, with gamma shape values of 0.3640 and 0.3970, respectively. The model of amino acids substitution for COI protein was the model described by Henikoff and Henikoff (1992) based on Block substitution matrices, BLOSUM62+I+G+F.

Maximum likelihood (ML) analyses, for both individual and concatenated genes, were conducted using the

software RAxML v. 7.0.4 (Stamatakis et al. 2008), and node support was assessed with nonparametric bootstrapping (BS) with 5,000 replicates, random starting trees and parameters estimated from each dataset under the model selected for the original dataset. Bayesian inference analyses (BI) were performed using the software MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). For these analyses, 6 substitution types (nst = 6) were used according to the evolutionary model determined by jModeltest. Analyses were initiated with random starting trees and run for 5,000,000 generations and four chains. The Markov chains were sampled each 1,000 generations. Two independent runs were performed. The program TRACER v1.5 (Lemey et al. 2009) was employed for determining when the log likelihood (ln l) of sampled trees reached a stationary distribution. Generations sampled before the chain reached stationary were discarded as burn-in. From the resulting trees, 500 were discarded (10 % of the generations) and the remaining trees of both runs were used to create 50 % majority-rule consensus tree and to estimate Bayesian posterior probabilities. Only nodes supported by BS $\geq$ 75 and PP $\geq$ 0.90 are discussed (Hillis and Bull 1993; Alfaro et al. 2003).

In order to compare the genetic distances among specimens of Polycerinae, we calculated the pairwise uncorrected *p*-distances for COI using PAUP* 4.0 b 10.0. All codon positions were considered for the analysis.

Results

The substitution saturation test for COI gene was not significant for Polycerinae, indicating that this gene was not saturated for substitutions within this subfamily. The saturation plots over divergence divided by codon for all dataset indicated that transversions were not saturated in the third position; therefore, nucleotides occupying this position were not excluded from the analyses. The genetic divergence (uncorrected *p*-distance) for COI within Polycerinae reached 19.5 % between some species of different genera (e.g., *Polycera* sp. B (Hawaii) and *Palio dubia*) (Table 2).

The ILD test showed no significant conflicting signal between the two genes. Consequently, a combined dataset was used for phylogenetic reconstruction. The trees obtained from the concatenated genes were very similar to those retrieved from COI and 16S genes independently, with the same strongly supported branches, with 16S being the least informative. On the other hand, the COI amino acid sequences were poorly informative (data not shown).

The combined dataset yielded a sequence alignment of 1,108 positions comprising 465 bp (16S) and 643 bp (COI, with 219 inferred amino acids).

The result of the maximum likelihood and Bayesian concatenated analyses is shown in Fig. 1, including the respective bootstraps supports values (BS) and posterior probabilities (PP). The monophyly of Polycerinae was strongly supported by both the maximum likelihood and the Bayesian analyses (BS = 100, PP = 1) with the genus *Crimora* as the sister taxon (BS = 85, PP = 1). Within Polycerinae, the relationships between genera were not resolved, originating a polytomy that included all the studied genera (*Thecacera*, *Polycerella*, *Palio,* and *Polycera*). Moreover, specimens of the genus *Gymnodoris* (*G.*

Table 2 Minimum COI gene pairwise uncorrected *p*-distances amongst some *Polycera* species

Species	COI genetic distances (%)
P. quadrilineata MNCN15.05/55455 (Sweden) versus *P. quadrilineata* MNCN15.05/55459 (Sweden)	0.0
P. capensis CASIZ176375 (SAfrica) versus *P. capensis* CASIZ176280 (SAfrica)	0.15
P. capensis CASIZ176907 (SAfrica) versus *P. capensis* CASIZ176280 (SAfrica)	0.64
P. capensis CASIZ176907 (SAfrica) versus *P. capensis* CASIZ176375 (SAfrica)	0.79
P. sp. A CASIZ176387 (SAfrica) versus *P.* sp. A CASIZ176169 (SAfrica)	1.40
P. faeroensis MNCN15.05/55503.1 (Portugal) versus *P. faeroensis* MNCN15.05/55503.2 (Portugal)	1.90
P. cf. *capensis* MNCN15.05/55470 (Australia) versus *P. capensis* CASIZ176280 (SAfrica)	2.18
P. sp. A CASIZ176169 (SAfrica) versus *P. capensis* CASIZ176375 (SAfrica)	9.64
P. sp. A CASIZ176169 (SAfrica) versus *P.* cf. *capensis* MNCN15.05/55470 (Australia)	10.1
P. quadrilineata MNCN15.05/55465 (Sweden) versus *P. faeroensis* MNCN15.05/55503.2 (Portugal)	12.3
P. tricolor CASIZ 76438a (California) versus *P. hedgpethi* MNCN15.05/55493 (Morocco)	12.8
P. sp. B CASIZ176795 (Hawaii) versus *P. capensis* CASIZ176375 (SAfrica)	13.1
P. sp. B CASIZ176795 (Hawaii) versus *P.* cf. *capensis* MNCN15.05/55470 (Australia)	14.0
P. hedgpethi MNCN15.05/55493 (Morocco) versus *Palio dubia* MNCN15.05/55467 (Sweden)	14.0
P. sp. C CASIZ120773 (Marshall Island) versus *P.* cf. *capensis* MNCN15.05/55470 (Australia)	14.9
P. sp. C CASIZ120773 (Marshall Island) versus *P. capensis* CASIZ176280 (SAfrica)	15.1
P. sp. B CASIZ176795 (Hawaii) versus *P.* sp. C CASIZ120773 (Marshall Island)	16.6
P. sp. C CASIZ120773 (Marshall Island) versus *P.* sp. A CASIZ176169 (SAfrica)	17.3
P. sp. C CASIZ120773 (Marshall Island) versus *P. faeroensis* MNCN15.05/55503.1 (Portugal)	18.2
P. sp. B CASIZ176795 (Hawaii) versus *Palio dubia* GB	19.5

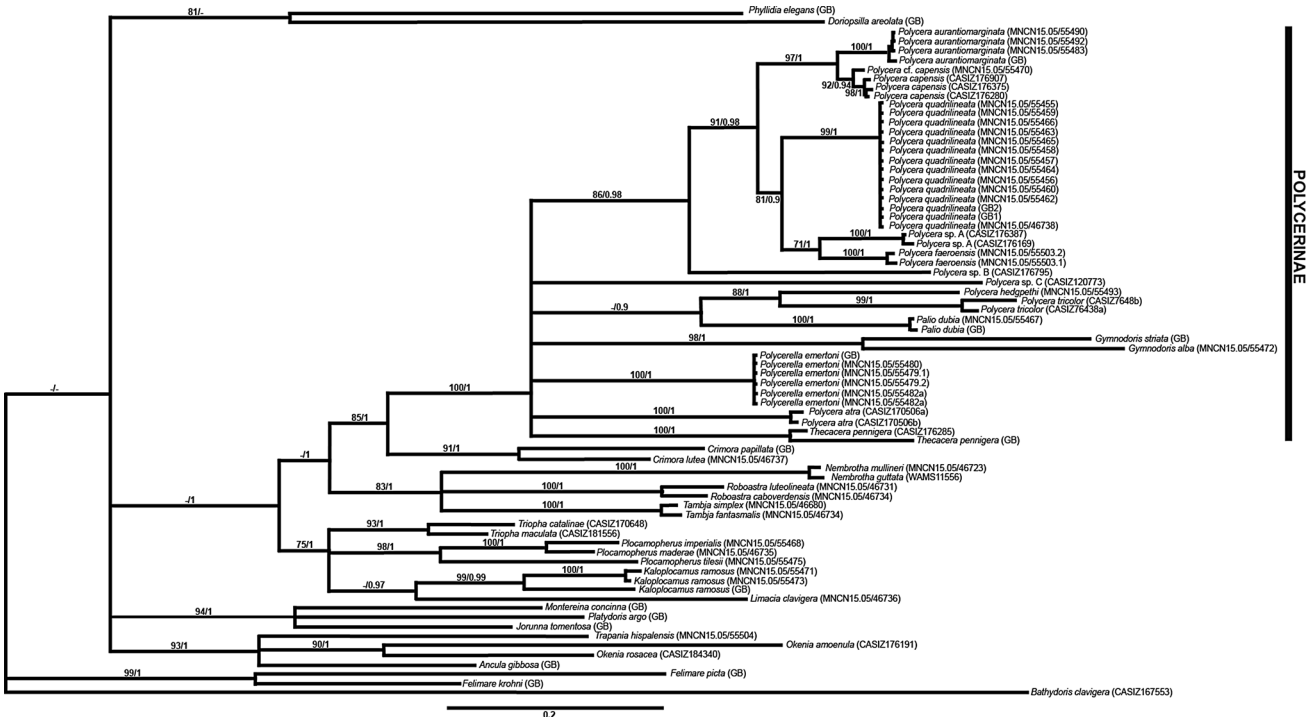

Fig. 1 Phylogenetic hypothesis based on combined molecular data (COI and 16S) represented by Bayesian inference. Numbers below the branches represent bootstraps supports values from the Maximum Likelihood analyses and posterior probabilities from Bayesian Inference, respectively (BS/PP). The *dashes* indicate bootstrap values below 75 and posterior probabilities below 0.9

alba and *G. striata*) clustered within Polycerinae (BS = 100, PP = 1).

The relationships within *Polycera* remained unresolved (Fig. 1). *Polycera tricolor* appeared sister to *Polycera hedgpethi* (BS = 88, PP = 1, minimum uncorrected *p*-distance = 12.8 %) and both bounded together with *Palio dubia*, but this relationship is not supported. *Polycera atra* formed an independent clade as well as the specimen *Polycera* sp. CASIZ 120773 from the Marshall Islands. The remaining *Polycera* were included in a strongly supported clade (BS = 86, PP = 0.98), with the specimen *Polycera* sp. CASIZ 176795 from Hawaii as sister to the rest of the taxa. This clade was divided into two well-supported subclades: one contained P. sp. from South Africa, as well as *P. quadrilineata* and *P. faeroensis* (BS = 81, PP = 0.9). All specimens of *P. quadrilineata* were grouped together regardless of their geographical origin with high posterior probability (1) and bootstrap value (99). The other subclade contained *Polycera aurantiomarginata*, *P.* cf. *capensis* from Australia, and three other specimens of *P. capensis* from South Africa (BS = 97, PP = 1). The COI genetic diverges between some of the studied specimens are summarized in Table 2.

The species considered from other subfamilies were grouped by genus, but the relationships between the subfamilies were not fully resolved.

Discussion

The monophyly of the subfamily Polycerinae is supported by the molecular data, confirming its phylogenetic identity as revealed by morphological studies (Alder and Hancock 1845; Odhner 1941). Unfortunately, the relationships within Polycerinae as well as between the studied species of *Polycera* remain unresolved. This result is probably explained by the fact that this study includes only a small representation of polycerid diversity. Including more species and nuclear markers may help to resolve this issue.

A controversial subject in current literature is the systematic position of *Gymnodoris*. Our data strongly support that *Gymnodoris* specimens analyzed (*G. alba* and *G. striata*) cluster within the subfamily Polycerinae with high bootstrap support and posterior probability values (Fig. 1). At present, these species are classified within an independent family, Gymnodorididae (Odhner 1941; Bouchet and Rocroi 2005), due to some differential traits like a different buccal apparatus and the capacity to feed on other nudibranchs (Macnae 1958; McDonald and Nybakken 1996). However, traditional classifications included gymnodorids as subfamily Gymnodoridinae within the family Polyceridae (Eliot 1903). Macnae (1958) considered that the differences between gymnodorids and polycerids were minor and the resemblances were too important to permit the separation in two families. Our results strongly support the

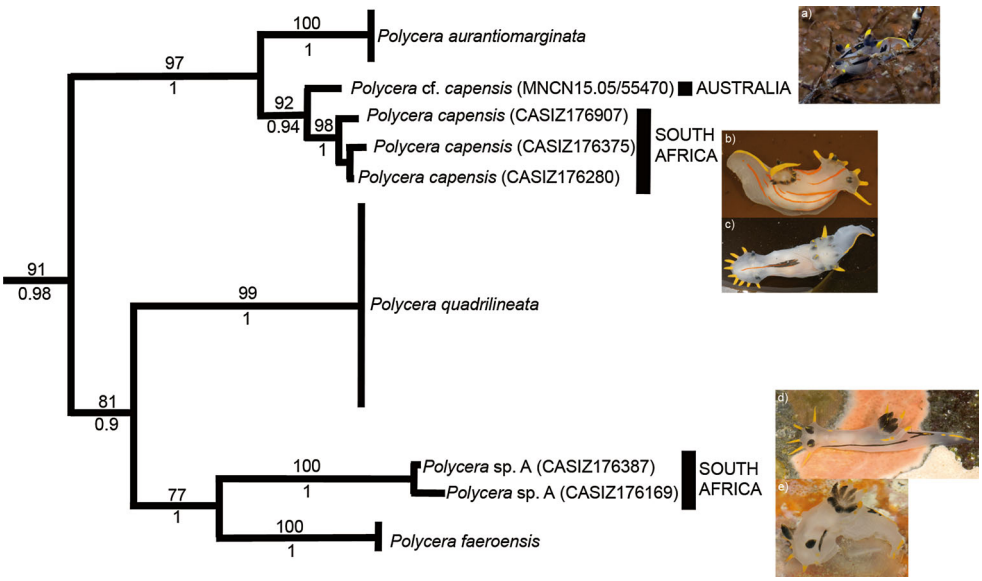

Fig. 2 Zoom of a branch of the bayesian inference tree of the concatenated dataset. *Numbers* below the branches represent bootstraps supports values (BS) from the maximum likelihood analyses and *numbers* under the branches represent posterior probabilities (PP). Photographs on the *right* represent **a** *P.* cf. *capensis* MNCN 15.05/55470, photo by Dave Harasti; **b** *P. capensis* CASIZ 176375, photo by T.M. Gosliner; **c** *P. capensis* CASIZ 176280, photo by T.M. Gosliner; **d** *P.* sp. A CASIZ 176387, photo by T.M. Gosliner; **e** *P.* sp. A CASIZ 176169, photo by T.M. Gosliner

old classification over the current one. However, although we strongly believe in our results, to date, Gymnodorididae comprises four genera and at least 19 species. We therefore think that more specimens are needed together with additional nuclear genes as well as further phylogenetic analysis to clarify the phylogenetic position of gymnodorids.

Within *Polycera* only *P. aurantiomarginata*, *P. capensis*, *P. quadrilineata*, *P. faeroensis*, two specimens of *P.* sp. A from South Africa (CASIZ176169 and CASIZ176387) and one specimen of an unidentified species (*Polycera* sp. B) formed a strongly supported clade. *P. faeroensis* appeared closely related to the South African *Polycera* sp. A and this clade is sister to *P. quadrilineata* (Fig. 2). *P. faeroensis* is morphologically similar to *P. quadrilineata* with some differences in cephalic processes (8 or more vs. 4–6, respectively). A close relationship between *P. quadrilineata* and *P. faeroensis* was mentioned by Odhner (1941), who postulated the separation between these species according to wing-like expansions in the jaws, while Lemche (1929) suggested that *P. faeroensis* was intermediate between the genera *Polycera* and *Palio*. Our results showed high genetic divergence between these two species (minimum uncorrected *p*-distance = 12.3 %), solving the controversy and corroborating the convenience of genetic analysis to improve taxonomic information obtained from morphological studies. On the other hand, the position of *P. aurantiomarginata* and *P. quadrilineata* in our study could fit with the morphological comparison of both species described by García and Bobo (1984). Their external morphology, radula and jaws are very similar; however, they differ in coloration of some specific parts, number of cephalic processes and lamellas of the rhinophores as well as some aspects of the larval development and spawning (García and Bobo 1984; Martínez-Pita et al. 2006).

In general, *Polycera* specimens were grouped by species with high bootstrap support and posterior probability values, regardless of the geographical origin of the sample (Fig. 1). An unexpected result was that some specimens from South Africa labeled in the field as *Polycera capensis* (CASIZ 176169 and CASIZ 176387) did not cluster together with the remaining *P. capensis* included on this study (three specimens from South Africa and one specimen from Australia) (Fig. 2). In fact, the minimum uncorrected *p*-distance for COI between specimens of these two different clades (10 %) clearly showed that they belong to different species (Table 2). In view of this result, we tried to re-examine the type material of *Polycera capensis,* but unfortunately "Le dessin original ná pas été conserve, non plus que l'échantillon" (Pruvot-Fol 1934). However, based on Quoy and Gaimards' original description of *Polycera capensis* and comparison with Quoy and Gaimard's plate of the type specimen, it seems more likely that the specimens here identified as a *P. capensis*-like animal from Australia are the true *P. capensis*. Allan (1932) described the Australian species known as *Polycera capensis* as a new and different species, *Polycera conspicua*. Later on, Pruvot-Fol (1934) re-named Allan's specimens as *Polycera capensis*; hence, *Polycera conspicua* became its synonym (Thompson 1975). Odhner (1941) supported this synonymy, but wrote about the differences between the different authors' pictures that he considered to be due to the morphological intraspecific variation. He thought that *Polycera capensis* had an Indian origin and later spread to the South. In 1958, Macnae also described variations in color and size of the mantle processes in spite of his agreement with the synonymy. In 1975, Thompson doubted that Pruvot-Fol (1934) and Odhner (1941) were correct in referring to Allan's specimens as *Polycera capensis*. Based on their

color form, the specimens described and figured by Allan (1932) from Sydney (Australia) as *P. conspicua* are also very similar to the specimen from Australia. We also dissected these specimens and compared them to the specimens studied by Macnae (1958), but since there are no pictures of Macnae's animals, we cannot be sure to which species the radula and the reproductive system showed in that paper belong.

Thus, one of the key findings of this study is that there are clearly at least two sympatric species of *Polycera* in South Africa, one more closely related to *P. faeroensis* and the other one more closely related to the *Polycera capensis*-like animal from Australia. It has been hypothesized that *Polycera capensis* was introduced from South Africa to Australia by fouling, and it has spread 200 km since then (Macnae 1958; Rudman 1998; Willan 2000). The maximum uncorrected *p*-distance for COI between the specimen of *Polycera capensis* from Australia and those from South Africa was 2.9 % (Table 2). Hebert et al. (2003) suggested a mean molluscan divergence of 11.1 ± 5.1 % for COI between sister species. Carmona et al. (2011) used maximum value of divergence for COI for the same species of 7.3 %, which is the value that they found between specimens of the same species from the Mediterranean Sea and the Western Atlantic. Wilson et al. (2009) even reduced this threshold to 5 % for well-supported sister pairs. Due to the fact that different groups of organisms, even within the same genus or family, can have different rates of molecular evolution (Hebert et al. 2003; Williams et al. 2003), and the use of general genetic thresholds to distinguish between species is difficult to apply. Therefore, we calculated a threshold based on our data. Within Polycerinae, individuals of the same species had a genetic divergence from 0 to 4 % for the COI gene. If this value were taken as threshold, specimens of *P. capensis* from Australia and from South Africa would be the same species as confirmed by our morphological studies.

Although our results suggest that the Australian specimen is conspecific with the South African ones, it is clear that *Polycera capensis* needs a deep morphological and molecular revision with larger sample sizes from Australia and South Africa to clarify this matter. Above all, a complete morphological redescription of *Polycera capensis* based on the study of material from the type locality is needed in order to objectively define this species and designate a neotype.

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