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Phylogenetic relationships of Nembrothinae (Mollusca: Doridacea: Polyceridae) inferred from morphology and mitochondrial DNA

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Abstract

Within the Polyceridae, Nembrothinae includes some of the most striking and conspicuous sea slugs known, although several features of their biology and phylogenetic relationships remain unknown. This paper reports a phylogenetic analysis based on partial sequences of two mitochondrial genes (cytochrome *c* oxidase subunit I and 16S rRNA) and morphology for most species included in Nembrothinae. Our phylogenetic reconstructions using both molecular and combined morphological and molecular data support the taxonomic splitting of Nembrothinae into several taxa. Excluding one species (*Tambja tentaculata*), the monophyly of *Roboastra* was supported by all the phylogenetic analyses of the combined molecular data. *Nembrotha* was monophyletic both in the morphological and molecular analyses, always with high support. However, *Tambja* was recovered as para- or polyphyletic, depending on the analysis performed. Our study also rejects the monophyly of "phanerobranch" dorids based on molecular data.

Keywords: Phylogeny; Taxonomy; Nembrothinae; COI; Mitochondrial DNA; 16S rRNA; Morphology; Total evidence

1. Introduction

The number of studies trying to resolve the phylogenetic relationships within dorid nudibranchs has increased markedly during the last few years, based on anatomical data and molecular analyses (Valdés and Gosliner, 1999, 2001; Wägele and Willan, 2000; Schrödl et al., 2001; Wollscheid-Lengeling et al., 2001; Valdés, 2002a, 2003; Fahey and Gosliner, 2004; Wägele and Klussmann-Kolb, 2005; Vonnemann et al., 2005). With about 2000 described species, the dorid nudibranchs constitute the most diverse group of opisthobranch mollusks. According to the currently accepted phylogenetic hypothesis (Wägele and Willan, 2000; Schrödl et al., 2001; Valdés, 2002a), *Bathydoris* is the sister group of the dorid nudibranchs and the most basal member of the clade Euctenidiacea. According to the nomenclature proposed in the recently published "Classification and Nomenclator of Gastropod Families" (Valdés and Bouchet, 2005), Doridacea includes Doridoidea [=Labiostomata] + Phyllidioidea [=Porostomata] + "Phanerobranchia". However, for the purpose of this paper we prefer to follow the nomenclature used in the classification proposed by Valdés (2002a). In this last classification Doridoidea + Phyllidioidea constitute Cryptobranchia, and Doridoidea includes Cryptobranchia + Phanerobranchia.

To date, most nudibranch phylogenetic studies have focused on cryptobranch dorids, which include more than 1500 species, while phylogenetic studies focused on the so-called "phanerobranch" dorids (about 500 species) are

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scarce. Regarding cryptobranch dorids, Valdés (2002a) found this group to be monophyletic, supported by several synapomorphies: labium present, dorsal gill retractable (when present), cavity around the anus, reduced number of oral tube and buccal bulb muscles and a differentiated prostate. Their phylogenetic relationships have also been investigated in previous morphological (Gosliner and Johnson, 1999; Fahey and Gosliner, 2001; Garovoy et al., 2001; Valdés, 2002a; Dorgan et al., 2002; Valdés and Behrens, 2002) and molecular (Thollesson, 2000; Fahey, 2003; Valdés, 2003) studies.

The main character that unites all "phanerobranch" dorids is the presence of a non-retractable dorsal gill. Nevertheless, Wägele and Willan (2000), Valdés (2002a), and Fahey and Gosliner (2004) were not able to identify synapomorphies to support this group and thus, "Phanerobranchia" appears to be paraphyletic. The phylogenetic hypotheses of the "Phanerobranchia" proposed by Vallés (2002), based on mitochondrial sequences of 14 taxa, offer only a partial resolution within this group. Moreover, the molecular analyses of the Nudibranchia, based on sequences of the 16S rRNA and cytochrome *c* oxidase subunit I mitochondrial genes (Thollesson, 1999a,b, 2000) were unable to recover precise phylogenetic relationships within this group. As in the case of cryptobranch dorids, the phylogenies of several "phanerobranch" groups, including Corambidae (Millen and Nybakken, 1991; Valdés and Bouchet, 1998), Goniodorididae (Gosliner, 2004), Aegiridae (Fahey and Gosliner, 2004) and the genus *Acanthodoris* (Fahey and Valdés, 2005), have been reconstructed. The most recent "phanerobranch" phylogeny shows that the traditional Onchidorididae is a paraphyletic group (Millen and Martinov, 2005).

Polyceridae Alder and Hancock, 1845 is one of the traditional groups of non-Suctoria "phanerobranch" dorids. It is comprised of four taxa considered as "subfamilies": Polycerinae, Triophinae, Nembrothinae and Kalinginae (Burn, 1967; Rudman, 1998). Ortea et al. (2004) recently proposed the new subfamily Kankelibranchinae, based on a single specimen from the coast of Cuba. Relationships among these subfamilies are poorly understood. Vallés (2002) presented a phylogenetic hypothesis of Triophinae, in which the subfamily appears to be paraphyletic due to the presence of *Kalinga ornata* as sister species to *Triopha*, based on morphological data.

The main objective of this study is to elucidate the phylogenetic relationships of the subfamily Nembrothinae Burn (1967). It is especially revelant to investigate the phylogeny of this group since members of the Nembrothinae show remarkable diversification of feeding biology, with various representatives feeding on bryozoans, tunicates and other Polyceridae. Understanding their phylogenetic relationships will shed light on their trophic specialization, ecological and biogeographical radiation on a global scale. Nembrothinae is composed of three genera: *Nembrotha* Bergh (1877), including 12 species with an Indo-Pacific distribution; *Roboastra* Bergh (1877) with six species (Pola et al., 2005a); and *Tambja* Burn, 1962 with 30 species, both latter genera distributed widely in tropical and temperate areas in the Atlantic, Eastern Pacific and Indo-Pacific (Pola et al., 2005b,c, 2006a,b). Molecular studies on this conspicuous, ecologically important and widely distributed group are scarce, and include only a few species within each "genus" (Pola et al., 2006a). In the present study, we analyze DNA sequences and morphological data of representative species of Nembrothinae from around the world. The mitochondrial genes selected are cytochrome *c* oxidase subunit I (COI) and 16S rRNA (16S). These markers have been used extensively and successfully for elucidating relationships among species and genera for different phyla (e.g., Avise, 1994), including other studies on opisthobranchs (Thollesson, 1999a,b, 2000; Medina and Walsh, 2000; Valdés, 2003; Grande et al., 2004). We also provide a new morphological matrix to provide the first total-evidence investigation of the Nembrothinae.

2. Materials and methods

2.1. Taxon sampling

Samples were obtained from specimens deposited in the collections of Museo Nacional de Ciencias Naturales, MNCN (Madrid), California Academy of Sciences, CASIZ (San Francisco), Muséum National d'Histoire Naturelle, MNHN (Paris), Australian Museum, AM (Sydney), Western Australian Museum, WAM (Perth), South Australian Museum, SAM (Adelaide), Instituto Nacional de la Biodiversidad, INBIO (Costa Rica) and the Zoologische Staatssammlung München, ZSM (Munich). The specimens used for molecular analyses, collection data, voucher information, and GenBank Accession Nos. are listed in Table 1. The specimens used for morphological analysis are listed in Table 2.

We obtained partial sequences of cytochrome *c* oxidase subunit I (658 bp) and 16S rRNA (479 bp) for 1 or 2 specimens of 26 species of Nembrothinae, 3 species of Triophinae and 1 species of Polycerinae. Another 6 sequences (2 Nembrothinae, 3 Polycerinae and 1 Goniodorididae) were obtained from GenBank. Seven species of cryptobranch dorids (two Porostomata: *Phyllidia elegans* and *Doriopsilla areolata*; and 5 Labiostomata: *Chromodoris krohni*, *Hypselodoris picta*, *Jorunna tomentosa*, *Discodoris concinna* and *Platydoris argo*) were obtained from GenBank. Species from GenBank were chosen based on the availability of partial sequences for both, the COI and 16S genes and also based on the availability of detailed morphological description for the same species. *Bathydoris clavigera* was chosen as the outgroup based on analyses by Wägele and Willan (2000), Schrödl et al. (2001) and Valdés (2002a), where the Bathydorididae was concluded to be the sister taxon to Doridoidea.

We used all available taxa (57) for the morphological phylogenetic analysis (Table 2). The morphological and anatomical features of these species were made directly

Table 1
Specimens used for molecular analyses, collection sites, vouchers, GenBank Accession Nos. and collectors

Species name	Locality	Voucher	GenBank Accession Nos.		Collector
			COI	16S	
**Roboastra europaea*	—	—	AY083457	AY083457	—
Roboastra caboverdensis	Cape Verde: Santo Antao Is.	MNCN 15.05/46614	EF142859	EF142908	M.A. Malaquias
Roboastra tigris	Mexico: Baja California	MNCN 15.05/46733	EF142860	EF142909	H. Bertsch
Roboastra luteolineata	Japan: Okinawa, Kerama Is., Zamami Is.	MNCN 15.05/46731	EF142861	EF142910	A. Ono
Roboastra luteolineata	Australia: Western Australia	WAM S23322	EF142862	EF142911	N. Wilson
**Roboastra luteolineata*	Australia: GBR, Heron Island	MNCN 15.05/46732	DQ231001	—	
Roboastra gracilis	Japan: Okinawa, Kerama Is., Zamami Is.	MNCN 15.05/46730	EF142863	EF142912	A. Ono
Tambja tentaculata	Guam: Apra Harbor, Western Shoals	MNCN 15.05/46681	EF142864	EF142913	M. Pola
Tambja tentaculata	Guam: Apra Harbor, Western Shoals	CASIZ 162639	—	EF142914	C. Carlson
Tambja olivaria	Philippines: Malapascua Is.,"Lapus-Lapus"	MNCN 15.05/46688	EF142865	EF142915	E. Köhler
Tambja affinis	Comores Island: Mayotte, Kongou	MNHN-Paris	EF142866	EF142916	M.Poddubetskaiaa
Tambja morosa	Japan: Okinawa, Kerama Is., Zamami Is.	MNCN 15.05/46673	EF142867	EF142917	A. Ono
Tambja morosa	Philippines: Cebu Is., Moalboal,"Pescador"	MNCN 15.05/46675	EF142868	EF142918	E. Köhler
Tambja verconis	Tasmania: Spring Beach	MNCN 15.05/46653	EF142869	EF142919	N. Wilson
Tambja verconis	Australia: NSW,Port Stephens,Nelson Bay Beach	MNCN 15.05/46654	—	EF142920	D. & L. Atkinson
Tambja sagamiana	Japan: Okinawa, Kerama Island, Amuro Is.	MNCN 15.05/46657	EF142870	EF142921	A. Ono
Tambja eliora	Mexico: Baja California Sur, Roca Suani	MNCN 15.05/46666	EF142871	EF142922	O. Angulo
Tambja eliora	Mexico: Baja California, Los Islotes	MNCN 15.05/46665	EF142872	EF142923	O. Angulo
**Tambja ceutae*	—	—	AY345038	AY345038	—
Tambja fantasmalis	Cape Verde: Boavista Is., Baía das Gatas	MNCN 15.05/46734	EF142873	EF142924	M. Malaquías
Tambja simplex	Cape Verde: Isla de San Vicente	MNCN 15.05/46680	EF142874	EF142925	G. Calado
Tambja capensis	South Africa, Cape Town, Bakoven	MNCN 15.05/46686	EF142875	EF142926	G. Calado
Tambja abdere	Mexico: Gulf of California, Bah.Banderas	MNCN 15.05/46659	EF142876	EF142927	A. Hermosillo
Tambja abdere	Mexico: Gulf of California, Bah.Banderas	MNCN 15.05/46658	—	EF142928	A. Hermosillo
**Tambja abdere*	—	—	DQ230995	—	
Tambja amakusana	Japan: Okinawa, Kerama Is., Zamami Is.	MNCN 15.05/46660	EF142877	EF142929	A. Ono
Tambja limaciformis	Japan: Okinawa, Kerama Is., Zamami Is.	MNCN 15.05/46689	EF142878	EF142930	A. Ono
Tambja gabrielae	Indonesia: N. Sulawesi, Lembeh Strait	CASIZ 162701	—	EF142931	C. Petrinos
Tambja blacki	Australia: Great Barrier Reef, Heron Is.	SAM D19352	EF142879	—	N. Wilson
Nembrotha chamberlaini	Philippines: Siguijor Island, Paliton Wall	MNCN 15.05/46727	EF142880	EF142932	E. Köhler
Nembrotha chamberlaini	Philippines: Siguijor Island, Paliton Wall	WAM S11566	EF142881	EF142933	E. Köhler
**Nembrotha chamberlaini*	—	—	DQ231006	—	
Nembrotha purpureolineata	Australia: NSW, Port Stephens	MNCN 15.05/46726	EF142882	EF142934	D. & L. Atkinson
Nembrotha purpureolineata	Australia: NSW, Port Stephens	C. 205326	EF142883	EF142935	D. & L. Atkinson

Nembrotha purpureolineata	Australia, Western Australia: Abrolhos Is.	WAM S11563	EF142884	EF142936	M. Pola
Nembrotha lineolata	Philippines: Negros Oriental Is., Lipayo	MNCN 15.05/46724	EF142885	EF142937	E. Köhler
Nembrotha lineolata	Japan: Okinawa, Kerama Island, Aka Is.	WAM S11562	EF142886	—	E. Köhler
**Nembrotha lineolata*	—	—	DQ231005	—	
Nembrotha megalocera	Egypt: Dahab, "coral-Garden"	MNCN 15.05/46729	EF142887	EF142938	M.Poddubetskaia
Nembrotha megalocera	Egypt: Dahab, Sinai	ZSM-Moll20006510	EF142888	EF142939	M. Schrödl
Nembrotha nigerrima	Philippines: Negros Oriental Is., Lipayo	WAM S11554	EF142889	EF142941	E. Köhler
Nembrotha nigerrima	Philippines: Cabilao Island, "Talisay Tree"	WAM S11553	EF142890	EF142940	E. Köhler
Nembrotha nigerrima	Japan: Okinawa, Kerama Island, Aka Is.	MNCN 15.05/46713	EF142891	—	A. Ono
Nembrotha cristata	Philippines: Apo Island, "Chapel"	MNCN 15.05/46714	EF142892	EF142942	E. Köhler
Nembrotha cristata	Philippines: Balicasag Is., Panglao Is.	MNCN 15.05/46715	EF142893	—	E. Köhler
Nembrotha guttata	Philippines, Siguijor Island, Siguijor Wall	WAM S11556	EF142894	EF142943	E. Köhler
Nembrotha mullineri	Philippines: Malapascua Is.,"Lapus-Lapus"	MNCN 15.05/46723	EF142895	EF142944	E. Köhler
Nembrotha milleri	Philippines: Negros Oriental Is., Lipayo	MNCN 15.05/46721	EF142896	—	E. Köhler
Nembrotha sp. 4	Australia: NSW, Port Stephens, Nelson Bay Beach	SAM D19354	EF142897	EF142945	N. Wilson
Nembrotha sp. 3	Indian Ocean: Comores Is. Mayotte	MNHN-Paris	EF142898	EF142946	M. Poddubetskaia
Nembrotha livingstonei	Philippines: Malapascua Island	MNCN 15.05/46716	EF142899	—	E. Köhler
Nembrotha cf. livingstonei	Japan: Okinawa, Kerama Island	MNCN 15.05/46717	EF142900	EF142947	A. Ono
Nembrotha sp. 1	Philippines: Negros Oriental Is., Lipayo	MNCN 15.05/46748	EF142901	EF142948	E. Köhler
Nembrotha sp. 2	Philippines: Negros Oriental Island, Danin	MNCN 15.05/46747	EF142902	EF142949	E. Köhler
Crimora lutea	Australia, Western Australia: Abrolhos Is.	MNCN 15.05/46737	EF142903	EF142950	M. Pola
Kaloplocamus ramosus	Portugal: Azores	—	EF142904	—	G. Calado
Plocamopherus maderae	Cape Verde Archipelago: Sal Island	MNCN 15.05/46735	EF142905	EF142951	P. Wirtz
Limacia clavigera	Spain: Bahía de Cádiz, Bajo Cabezuela	MNCN 15.05/46736	EF142906	EF142952	J. L. Cervera
Polycera quadrilineata	Escócia: D.M.L. Oban	MNCN 15.05/46738	EF142907	EF142953	J. L. Cervera
**Polycera quadrilineata*	—	—	AJ223275	AJ225200	
**P. aurantiomarginata*	—	—	AJ223277	AJ225199	
**Thecacera pennigera*	—	—	AJ223277	AJ225202	
**Ancula gibbosa*	—	—	AJ223255	AJ225179	
**Platydoris argo*	—	—	AY345037	AY345037	
**Discodoris conccina*	—	—	AF249801	AF249228	
**Jorunna tomentosa*	—	—	AJ223267	AJ225191	
**Hypselodoris picta*	—	—	AF249787	AF249238	
**Chromodoris krohni*	—	—	AY345036	AY345036	
**Phyllidia elegans*	—	—	AJ223276	AJ225201	
**Doriopsilla areolata*	—	—	AJ223262	AJ225186	
**Bathydoris clavigera*	—	—	AF249808	AF249222	

* Directly retrieved from GenBank.

Table 2
Species included in the morphological analysis, with the sources of information

Taxa	Source of information
Bathydoris clavigera (Thiele, 1912)	Wägele (1989), Wägele and Willan (2000), Valdés (2002b), Fahey and Gosliner (2004)
Doriopsilla areolata (Bergh, 1880)	Valdés and Ortea (1997), García-Gómez (2002)
Platydoris argo (Linné, 1767)	García and García-Gómez (1989), Valdés and Gosliner (2001), Dorgan et al. (2002)
Phyllidia elegans (Bergh, 1869)	Directly from the available material, Brunckhorst (1993)
Jorunna tomentosa (Cuvier, 1804)	Valdés and Gosliner (2001), García-Gómez (2002)
Discodoris concinna (Gould, 1852)	Gohar and Soliman (1967), Valdés and Templado (2002)
Chromodoris krohni (Vérany, 1846)	García-Gómez (2002), Ortea et al. (1996)
Hypselodoris picta (Schultz in Philippi, 1836)	Ortea et al. (1996), Gosliner and Johnson (1999)
Ancula gibbosa (Risso, 1818)	Baba (1990), Gosliner (1994), Valdés (2002b)
Polycera quadrilineata (Müller, 1776)	Thompson and Brown (1984), García-Gómez (2002)
Polycera aurantiomarginata García-Gómez and Bobo (1984)	García-Gómez and Bobo (1984), García-Gómez (2002), Vallès et al. (2000)
Polycerella emertoni (Verrill, 1880)	Directly from the available material, Behrens and Gosliner (1988), García-Gómez and Bobo (1986)
Thecacera pennigera (Montagu, 1815)	Marcus (1957), Willan (1976, 1989),
Limacia clavigera (Müller, 1776)	Directly from the available material, García-Gómez (2002), Schmekel and Portmann (1982), Ortea et al. (1989)
Plocamopherus maderae (Lowe, 1842)	Directly from the available material, Eliot (1908).
Roboastra species	Pola et al. (2003, 2005a).
Tambja species	Pola et al. (2005b,c, 2006a,b,c)
Nembrotha species	Pola et al. (submitted for publication)

from the available material. In some cases, the complete published description of certain features of a species allowed the extraction of data from the literature, which was then verified by direct examination of a specimen, whenever available (Table 2). The cryptobranch dorids were included in the analysis for comparative purposes and to test the monophyly of the Phanerobranchia. The genera were chosen from the analysis of Valdés (2002a). In the case of the "phanerobranch" dorids, there are few phylogenies available (Fahey and Gosliner, 2004). To date, no phylogenetic analysis has been undertaken for members of *Nembrotha*. Recent studies including some species of this taxon (Pola et al., 2005a, 2006c) were solely based on morphological data. For the morphological analysis, one to ten specimens per species were used to define the characters that diagnose that species.

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from small amounts of ethanol-preserved tissues (muscle from the foot or mantle except in those cases of small animals where the whole specimen was used). Tissues were lysed in 600 μl of homogenizing buffer (CTAB: 2%, ClNa: 1.4 M, Bmercaptoethanol: 0.2%, EDTA: 20 mM, Tris: 0.1 M pH 8) and digested with 8 μl of Proteinase K (20 mg/ml) for 48 h at 55 °C. After homogenization, total cellular DNA was purified by a standard phenol:dichloromethane:iso-amyl alcohol (24:1) protocol (Winnipenninckx et al., 1993).

Partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) and mitochondrial large ribosomal subunit (16S rRNA) were amplified by polymerase chain reaction (PCR) using the following primers: 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′) (Folmer et al., 1994) for COI and 16Sar-L (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16Sbr-H (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Palumbi et al., 1991) for 16S rRNA.

Standard PCRs for COI amplification consisted of 40 cycles with a denaturing temperate of 94 °C (1 min), annealing at 46°–48° (1 min) and extension at 72 °C (1 min). An 8-min elongation step at 72 °C completed the reaction. The partial 16S amplification followed the same conditions, except for 35 cycles and a higher annealing temperate (48°–50°). PCRs were performed in a total volume of 25 μl, including 1 U of *Taq* Polymerase (Biotools), 1 μl of each primer, 1 μl of dNTPs, 0.5 μl $MgCl_2$ (25 mM) plus 2.5 μl of a reaction buffer (Biotools). Double-stranded amplified product was electrophoresed in a 1.5% agarose gel and cleaned using ethanol precipitation, resuspending in 22 μl of ddH_2O. The amplified fragments were sequenced in both directions in an automated DNA sequencer (Applied Biosystems Prism 3700) following the manufacturer's instructions.

2.3. Sequence alignment and analyses

The forward and reverse DNA sequences were assembled and checked against each other using Sequence Navigator version 1.0.1 (Applied Biosystems). Clustal X (Aladdin Systems, Heidelberg, Germany) was employed to align the 16S gene sequences and then refined manually by comparing them to published secondary structure models for 16S rRNA (Thollesson, 1999b; Valdés, 2003). Additionally, all alignments were checked visually. To choose among alternative alignments, they were checked by

performing parsimony searches, and the alignment that yielded the shortest tree was then chosen.

2.4. Morphological data

The morphological dataset comprised 49 characters for 57 species, including the outgroup taxon. The description of the characters and their states is shown in Appendix 1; the morphological data matrix is shown in Appendix 2. The specimens were dissected by dorsal incision to facilitate morphological examination. The internal features were examined using a dissecting microscope with a camera lucida attached. Special attention was paid to the morphology of the reproductive system, including the penial hooks. The penis was critical point dried for scanning electron microscopy. The buccal mass was dissolved in 10% sodium hydroxide until the radula was isolated from the surrounding tissue. The radula was then rinsed in water, dried and mounted for examination by scanning electron microscopy. No color details were considered as characters in this study.

2.5. Phylogenetic analyses

Saturation among sequences was plotted by category of substitution (transitions and transversion) and by first, second and third codon position to test for the possibility that some types of nucleotide substitutions have become saturated. Sequence analysis was based on the parsimony (MP) and maximum likelihood (ML) optimality criteria. The evolutionary model that best fit our data was selected using MODELTEST 3.06 (Posada and Crandall, 1998) under the Akaike information criterion (Akaike, 1974). Parsimony analyses were performed by heuristic searches under TBR branch swapping and 100 random replicates using the PAUP* 4.0b10 (Swofford, 2002). We evaluated the effect of using gaps as missing data and as a fifth character state, which increased nodal support of most nodes (see Giribet and Wheeler, 1999). Parsimony analyses were performed with all characters unweighted for morphological, molecular and combined molecular data sets.

Maximum likelihood analyses were also run in PAUP* for the molecular data only, through heuristic searches, with the same parameters indicated above and using the model selected by MODELTEST. The model and the associated parameters selected by MODELTEST and applied in the ML analysis for each molecular dataset are shown in Table 3. We used nonparametric bootstrapping (1000 pseudoreplicates) (bs) in the MP to assess the nodal support (Felsenstein, 1985; Felsenstein and Kishino, 1993). Nonparametric bootstrapping implemented in Phyml (Guindon and Gascuel, 2003) was used for the ML analyses using 500 pseudoreplicates. Shimodaira–Hasegawa parametric tests (Shimodaira and Hasegawa, 1999) using bootstrap with full optimization (1000 bs), were used to test for the monophyly of selected taxa (Leaché and Reeder, 2002) as implemented in PAUP*. Initially, all analyses were performed excluding and including third codon positions. However, since extensive evidence indicates that third codon positions are of great value precisely because of their high and differential rate (Yoder et al., 1996; Lewis et al., 1997; Björklund, 1999; Källersjö et al., 1999; Baker et al., 2001) we decided to include all positions in our analyses.

Table 3
Model and the associated parameters selected by MODELTEST and applied in the ML analysis for each molecular dataset

Gen	16S	COI	16S+COI
Model	TIM+I+G	GTR+I+G	GTR+I+G
Base frequencies			
A	0.3654	0.2929	0.3327
C	0.0903	0.0965	0.0849
G	0.1715	0.1307	0.1498
T	0.3728	0.4799	0.4326
Rate matrix			
A	1.0000	0.5949	1.3701
C	5.4263	22.6710	11.2481
G	1.3939	0.6480	1.132
T	8.8758	53.8200	3.872
∝	0.4201	0.3529	0.4447
I	0.3666	0.4495	0.4643
g1 ($P < 0.01$)	−0.33	−0.53	−0.46
Mean ± SD tree length	1737.476 ± 38.24	4618.855 ± 72.05	5472.045 ± 86.92

The analyses were performed using the combined molecular data sets but each gene was also analyzed independently. For the 16S rRNA locus, two types of tests were run in which we either took into account or did not consider the most variable regions (in which alignments between separate groups were most difficult). The secondary structure diagram of *Thecacera pennigera* (Montagu, 1815) by Thollesson (1999b) and *Dendrodoris denisoni* (Angas, 1864) by Valdés (2003) were used as the structure model for identifying loop regions. These ambiguously aligned regions were finally removed from the analysis. A total of 63 nucleotides were excluded from the analysis, in the following regions: 888–913; 950–974; 982–993.

Incongruence length differences (ILD) tests (Farris et al., 1994) were conducted using the partition homogeneity test in PAUP* to determine the congruence between the different sets of data (Cunningham, 1997). Test settings consisted of 10 random stepwise additions (100 replicates) with TBR branch swapping. Analyses were performed using parsimony (with the heuristic search option) as the optimality criterion.

We also performed a Bayesian analysis to estimate the posterior probabilities of the nodes in the phylogenetic tree. Mr Bayes 3.0b3 (Huelsenbeck and Ronquist, 2001) was run with 6 substitution types (nst = 6). This procedure is based on a GTR model and considers gamma distributed rate variation as well as the proportion of invariable positions for the two genes combined. Data from each of the two genes were treated as different data partitions. Analyses were initiated with random starting trees and run for

2,000,000 generations. The Markov chains were sampled each 100 generations. Of the resulting trees, 2100 were discarded as "burn in".

Morphological data were analyzed under the parsimony criterion. Characters were treated as unordered and unweighted. The parsimony analysis was conducted in PAUP by heuristic search under TBR branch swapping and random taxon addition (10 replicates), finding the consensus tree among the equally parsimonious tree obtained. Branch support was assessed with nonparametric bootstrapping (50 replicates).

3. Results

3.1. Sequence characteristics and variation

As indicated in Table 4, amplifications were not successful for some gene fragments in isolated taxa. After alignment, 1074 bp were used: 658 for COI and 416 for 16S. Non-aligned 16S sequences were 479 bp in length. The three most variable regions occurring between positions 230 and 255, 292 and 316 and between 324 and 335 were excluded. We obtained 46 new sequences for 16S and 51 for COI, and used 4 COI sequences from a previous study (Pola et al., 2006a) and 14 sequences from GenBank (only two of them of Nembrothinae). Finally, we managed to obtain sequences for both genes in 56 specimens (Table 4). Base composition was homogeneous in all the taxa analyzed except for third codon positions (Table 5). Both gene fragments showed an AT bias, especially in COI third codon positions. The saturation plots of absolute differences against corrected sequence divergences divided by codon indicated no saturation when we plotted all the substitutions together, but signs of a saturation tendency were shown for transitions in third codon positions of the COI gene for divergences values above 0.15 (or 15%) (Fig. 1). The exclusion of the third codon positions did not change the topology of the trees (not shown). The second codon was extremely conservative, with up to four substitutions (transitions or transversions) found in comparisons between Nembrothinae and outgroup taxa. Out of the 1.541 pairwise substitutions screened, only one pair, *Ancula gibbosa* vs. *Polycera aurantiomarginata*, showed ten substitutions in total (adding transitions and transversions). Direct estimation of the transition/transversion ratio gave a higher value for COI (ts/tv ratio = 9.98) than for 16S (ts/tv ratio = 2.11). Within Nembrothinae, sequence divergence for both genes ranged from 0.24% or 16.64% (between 16S sequences of *Roboastra europaea* vs. *R. caboverdensis* and *N.* sp. 4 vs. *Tambja limaciformis*, respectively) to 25.54% (intergeneric divergence of COI between *Nembrotha chamberlaini* and *Roboastra gracilis*). The mean values were similar across taxa and almost always several times higher for COI than for 16S. Nevertheless, we found some discordant cases. Within the *Nembrotha* samples, two specimens considered as *N.* sp. 1. and *N.* sp. 2. (based on external colouration differences) showed 0.00% divergence in the 16S gene between them and with *N. cristata* and 0.46% (*N.* sp. 1 vs. *N. cristata*), 0.77% (*N.* sp. 2 vs. *N. cristata*) and 0.92% (*N.* sp. 2 vs. *N.* sp. 1) in COI.

3.2. Phylogenetic relationships based on molecular data

The non-monophyly and the relationships among cryptobranch dorids are not discussed in this paper. The monophyly of the traditional group "phanerobranch" is rejected by all our molecular phylogenetic analyses since *Ancula gibbosa* appears more closely related to cryptobranch than to "phanerobranch" dorids. Moreover, our data provided no evidence for recognition of the family Polyceridae, which is not monophyletic under any molecular sets. Additional cryptobranchs and "phanerobranch" taxa must be included in the analyses for a better assessment of their relationships.

3.2.1. Single genes (16S rRNA and COI sequences)

The parsimony analyses produced six equally parsimonious trees [788 steps; consistency index (CI) = 0.4, retention index (RI) = 0.6] for the 16S analysis and 773 equally parsimonious trees [2347 steps; consistency index (CI) = 0.2, retention index (RI) = 0.6] for the COI analysis when all characters were weighted equally and gaps were interpreted as five positions (trees not shown). Regarding the 16S sequences, the monophyletic ingroup (ML = 78, Pp = 0.99, MP = 73) showed two different topologies based on different analyses; in the Bayesian and MP trees, *Tambja capensis* occupied a basal position, being sister taxon to the remaining species of Nembrothinae. However, the ML non-parametric bootstrap analysis recovered that *T. capensis* was included within the clade containing *Tambja* + *Roboastra* species. Nevertheless, the bootstrap value of this latter clade was low and this resulted in a basal polytomy that included seven major clades: (1) a clade containing *T. tentaculata* and *Roboastra* species, (2) a clade containing *T. amakusana*, *T. limaciformis*, (3) a clade containing *T. fantasmalis* and *T. simplex*, (4) *T. abdere*, (5) a clade containing *T. capensis*, (6) a clade containing *T. olivaria*, *T. affinis*, *T. morosa*, *T. gabrielae*, *T. verconis*, *T. sagamiana*, *T. ceutae* and *T. eliora* and (7) a clade containing *Nembrotha* species. The COI analysis did not recover the monophyly of Nembrothinae. The monophyly of *Nembrotha* was supported in all the analytical methods with very high support values for both, 16S and COI sequences. *Nembrotha* split into two major divisions: one including *N. megalocera*, *N.* sp. 3, *N. purpureolineata*, *N. lineolata* and *N. chamberlaini*, and a clade including *N. nigerrima*, *N.* cf. *livingstonei*, *N. guttata*, *N. cristata*, *N. mullineri*, *N.* sp. 4, *N.* sp. 1 and *N.* sp. 2. Based on 16S sequences, *Roboastra* appeared as non-monophyletic since the relationship with *R. gracilis* was not supported. The clade containing *R. europaea*, *R. caboverdensis*, *R. luteolineata* and *R. tigris* was well-supported (ML = 100, Pp = 0.98, MP = 99) with *R. tigris* being the sister taxon of that group. Based on the COI sequences *Roboastra* also appeared as non-monophyletic

Table 4
Samples included on each dataset for analyses (√=data available; X=data not available)

Species	Voucher	Morp.	COI	16S	COI+16S	Total
Roboastra europaea	—	√	√	√	√	√
Roboastra caboverdensis	MNCN15.05/46614	√	√	√	√	√
Roboastra tigris	MNCN15.05/46733	√	√	√	√	√
Roboastra luteolineata	MNCN15.05/46731	√	√	√	√	√
Roboastra luteolineata	WAM S23322	√	√	√	√	√
Roboastra luteolineata	MNCN15.05/46732	√	√	X	X	√
Roboastra gracilis	MNCN15.05/46730	√	√	√	√	√
Roboastra leonis	CASIZ 097577	√	X	X	X	√
Tambja tentaculata	CASIZ 162639	√	X	√	X	√
Tambja tentaculata	MNCN15.05/46681	√	√	√	√	√
Tambja olivaria	MNCN15.05/46688	√	√	√	√	√
Tambja affinis	MNHN-Paris	√	√	√	√	√
Tambja morosa	MNCN15.05/46673	√	√	√	√	√
Tambja morosa	MNCN15.05/46675	√	√	√	√	√
Tambja verconis	MNCN15.05/46653	√	√	√	√	√
Tambja verconis	MNCN15.05/46654	√	X	√	X	√
Tambja sagamiana	MNCN15.05/46657	√	√	√	√	√
Tambja eliora	MNCN15.05/46666	√	√	√	√	√
Tambja eliora	MNCN15.05/46665	√	√	√	√	√
Tambja eliora	—	X	√	X	X	X
Tambja ceutae	MNCN15.05/46661	√	√	√	√	√
Tambja fantasmalis	MNCN15.05/46734	√	√	√	√	√
Tambja simplex	MNCN15.05/46680	√	√	√	√	√
Tambja capensis	MNCN15.05/46686	√	√	√	√	√
Tambja abdere	MNCN15.05/46659	√	√	√	√	√
Tambja abdere	MNCN15.05/46658	√	X	√	X	√
Tambja abdere	MNCN15.05/46742	√	√	X	X	√
Tambja amakusana	MNCN15.05/46660	√	√	√	√	√
Tambja limaciformis	MNCN15.05/46689	√	√	√	√	√
Tambja oliva	INB0003348523	√	X	X	X	√
Tambja mullineri	CASIZ 067100	√	X	X	X	√
Tambja marbellensis	MNCN15.05/26031	√	X	X	X	√
Tambja victoriae	CASIZ 075810	√	X	X	X	√
Tambja zulu	CASIZ 074085	√	X	X	X	√
Tambja gabrielae	CASIZ 162701	√	X	√	X	√
Tambja stegosauriformis	MZSP 44650	√	X	X	X	√
Tambja tenuilineata	MNCN15.05/46687	√	X	X	X	√
Tambja blacki	SAM D19352	√	√	X	X	√
Tambja haidari	MNCN15.05/46710	√	X	X	X	√
Nembrotha chamberlaini	MNCN15.05/46727	√	√	√	√	√
Nembrotha chamberlaini	WAM S11566	√	√	√	√	√
Nembrotha chamberlaini	—	X	√	X	X	X
Nembrotha purpureolineata	MNCN15.05/46726	√	√	√	√	√
Nembrotha purpureolineata	C. 205326	√	√	√	√	√
Nembrotha purpureolineata	WAM S11563	√	√	√	√	√
Nembrotha lineolata	MNCN15.05/46724	√	√	√	√	√
Nembrotha lineolata	WAM S11562	√	√	X	X	√
Nembrotha lineolata	CASIZ 158257	√	√	X	X	√
Nembrotha megalocera	MNCN15.05/46729	√	√	√	√	√
Nembrotha megalocera	ZSMMoll20006510	√	√	√	√	√
Nembrotha nigerrima	WAM S11554	√	√	√	√	√
Nembrotha nigerrima	WAM S11553	√	√	√	√	√
Nembrotha nigerrima	MNCN15.05/46713	√	√	X	X	√
Nembrotha cristata	MNCN15.05/46714	√	√	√	√	√
Nembrotha cristata	MNCN15.05/46715	√	√	X	X	√
Nembrotha guttata	WAM S11556	√	√	√	√	√
Nembrotha mullineri	MNCN15.05/46723	√	√	√	√	√
Nembrotha milleri	MNCN15.05/46721	√	√	X	X	√
Nembrotha sp. 4	SAM D19354	√	√	√	√	√
Nembrotha sp. 3	MNHN-Paris	√	√	√	√	√
Nembrotha livingstonei	MNCN15.05/46716	√	√	X	X	√
Nembrotha cf. livingstonei	MNCN15.05/46717	√	√	√	√	√
Nembrotha sp. 1	MNCN15.05/46748	√	√	√	√	√

(*continued on next page*)

Table 4 (*continued*)

Species	Voucher	Morp.	COI	16S	COI+16S	Total
Nembrotha sp. 2	MNCN15.05/46747	√	√	√	√	√
Crimora lutea	MNCN15.05/46737	X	√	√	√	√
Kaloplocamus ramosus	—	X	√	X	X	√
Plocamopherus maderae	MNCN15.05/46735	√	√	√	√	√
Limacia clavigera	MNCN15.05/46736	√	√	√	√	√
Polycera quadrilineata	MNCN15.05/46738	√	√	√	√	√
Polycera quadrilineata	—	√	√	√	√	√
Polycera aurantiomarginata	—	√	√	√	√	√
Polycerella emertoni	—	√	X	X	X	√
Thecacera pennigera	—	√	√	√	√	√
Ancula gibbosa	—	√	√	√	√	√
Platydoris argo	—	√	√	√	√	√
Discodoris conccina	—	√	√	√	√	√
Jorunna tomentosa	—	√	√	√	√	√
Hypselodoris elegans	—	√	√	√	√	√
Chromodoris khroni	—	√	√	√	√	√
Phyllidia elegans	—	√	√	√	√	√
Doriopsilla areolata	—	√	√	√	√	√
Bathydoris clavigera	—	√	√	√	√	√

Table 5
Number of characters analyzed, nucleotide proportions, and transition/transversion (ts/tv) ratios for all the taxa analyzed according to COI and 16S rRNA sequences

COI					16S
Codon position	1st	2nd	3rd	All	
Characters					
Total	219	219	220	658	416
Constant	158	199	4	361	223
(%)	72.15	90.87	1.82	54.86	53.6
Parsimony informative	51	8	212	271	139
(%)	23.3	3.65	96.36	41.2	33.4
A (%)	24.45	12.50	32.32	23.10	30.83
C (%)	15.28	24.61	7.08	15.65	15.59
G (%)	29.36	18.91	13.19	20.48	22.06
T (%)	30.91	43.98	47.41	40.77	31.52
P	1.00	1.00	0.00	0.99	1

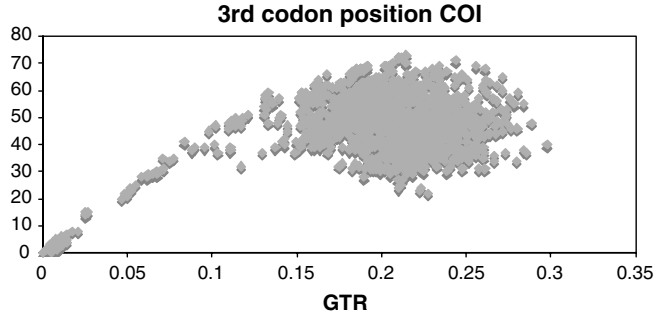

Fig. 1. Saturation plot: relationships between corrected mean divergences (GTR) between pairs of taxa and the number of transitional changes in the third codon position of the COI sequence.

genus since the clade containing *Roboastra* + *Tambja tentaculata* was highly supported in most of the analytical methods (ML = 82; Pp = 0.98, not recovered with MP). Finally, *Tambja* clustered in different groups, with uncertain phylogenetic relationships.

3.2.2. Combined molecular data (16S, COI)

Despite the differences stated, the partition homogeneity test (ILD test) indicated no significant differences in the topologies obtained separately from each gene ($P > 0.05$). The parsimony analysis yielded twelve most parsimonious trees ($L = 3034$ steps; 412 characters were parsimony informative; CI = 0.3, RI = 0.6) when all characters were weighted equally and gaps were interpreted as five positions.

The maximum likelihood analysis of the combined molecular data set resulted in a tree (lnL = −13348.709) where Nembrothinae formed a monophyletic group (ML = 89, Pp = 1.00, MP = 67) (Fig. 2). Within Nembrothinae, the genus *Nembrotha* was monophyletic with very high support (ML = 100, Pp = 1.00, MP = 99) and sister to a clade formed by *Tambja* + *Roboastra* species (ML = 53, Pp = 0.89, not recovered with MP).

Regarding *Nembrotha*, two clades were recovered, both supported by high probability values in all the analytical methods (Fig. 2). One clade included *N. livingstonei* and *N. nigerrima* sister to that integrated by *N. cristata*, *N. guttata*, *N. mullineri*, *N.* sp. 4, *N.* sp. 1 and *N.* sp. 2. In the other clade, *N. megalocera* is sister species to an assemblage formed by *N. chamberlaini*, *N. purpureolineata*, *N. lineolata* and *N.* sp. 3.

In the clade *Tambja* + *Roboastra* five major monophyletic groups were shown (Fig. 2). The first branch of this subclade was that including *Tambja abdere*, *T. amakusana* and *T. limaciformis*, with *T. abdere* as the more basal of the three species and *T. amakusana* and *T. limaciformis* as sister species. The clade including *T. amakusana* and *T. limaciformis* was supported by high probability values (ML = 100, Pp = 1.00, MP = 100) while the relationship of it with *T. abdere* showed low support (Pp = 0.51, not recovered with MP or ML). Therefore, there was a basal polytomy resulting in six major clades instead the five mentioned above. *Tambja capensis* formed the third clade, basal to that containing the remaining species of *Tambja*

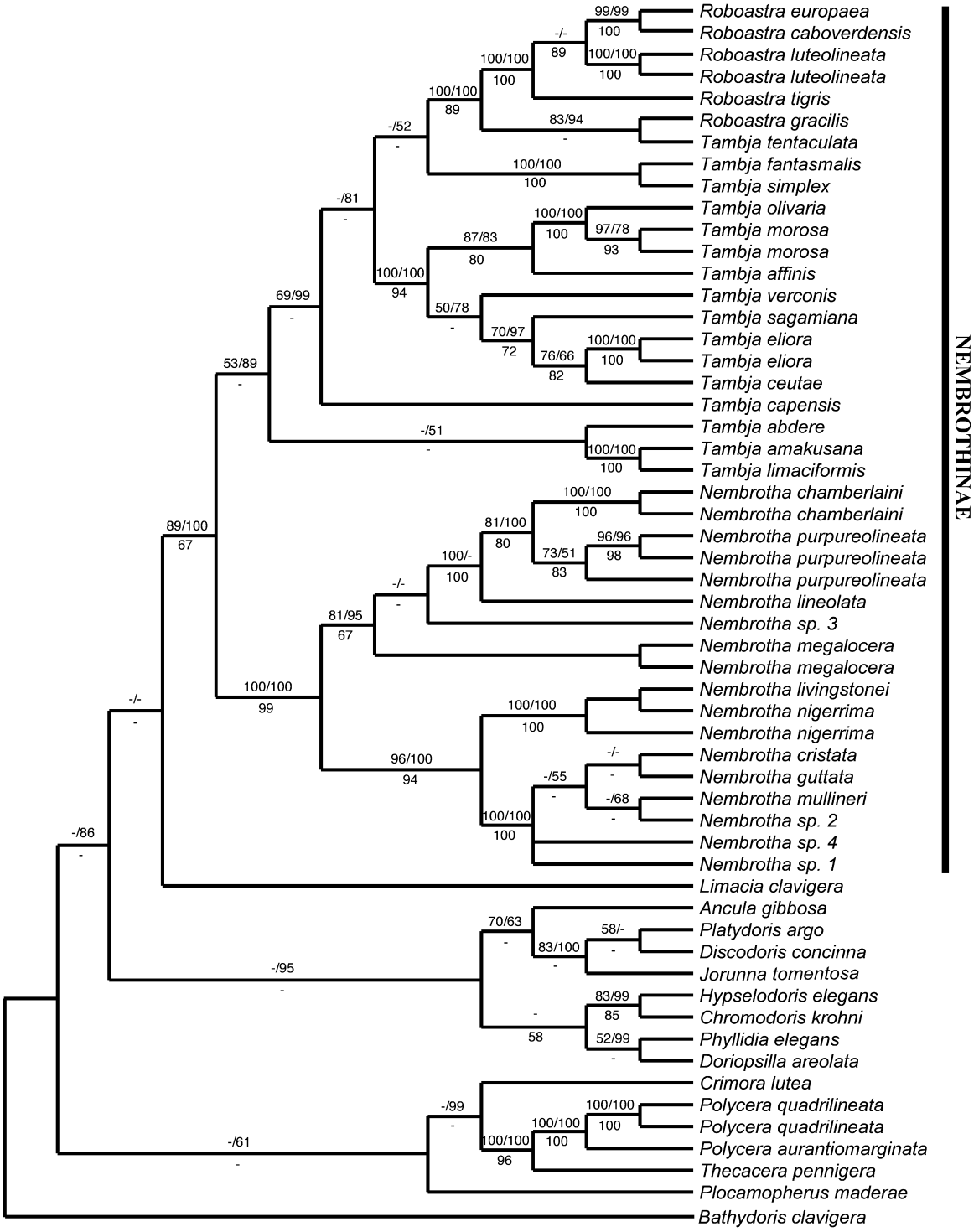

Fig. 2. Phylogenetic hypothesis based on combined molecular data (COI+16S) represented by Bayesian inference. Numbers above branches represent those in first position bootstrap values for ML, and number in second position, posterior probabilities from Bayesian inference. When numbers appear under branches, they indicate bootstrap values for MP.

and *Roboastra*. The fourth group was comprised of seven species of *Tambja*, including the type species of the genus, *Tambja verconis*. This clade was very well supported in all analyses (ML = 100, Pp = 1.00, MP = 94). Within this clade, there was one group including *T. olivaria* and *T. morosa* as sister species and *T. affinis* basal to them and other group with *T. eliora* and *T. ceutae* as sister species, *T. sagamiana* sister to the latter and *T. verconis* as the more basal of the four species. The fifth group included *T. fantasmalis* and *T. simplex*.

Finally, the monophyly of the genus *Roboastra* was broken by the species *Tambja tentaculata* that appears sister to

Roboastra gracilis. This clade [*Roboastra* + *T. tentaculata*] was very well supported (ML = 100, Pp = 1.00, MP = 89). The remaining species of *Roboastra* form a clade in which *R. tigris* is basal to *R. luteolineata*, *R. europaea* and *R. caboverdensis*, being *R. europaea* and *R. caboverdensis* sister species.

When we forced all the samples of *Tambja* to form a monophyletic group, the tree obtained ($\ln L = -13919.202$) differs significantly ($P < 0.05$) from the tree shown in Fig. 2, based on Shimodaira–Hasegawa parametric test (Shimodaira and Hasegawa, 1999).

3.3. Phylogenetic relationships based on morphological data

From the analysis of the data matrix, 1170 most parsimonious trees, each 159 steps long were obtained, with a consistency index of 0.34 and retention index of 0.83.

The strict consensus of those trees (Fig. 3) showed monophyly of Cryptobranchia, "Phanerobranchia", Polyceridae, Nembrothinae, *Roboastra* and *Nembrotha*. However, the monophyly of the subfamily Polycerinae was not supported and the genus *Tambja* was depicted as polyphyletic.

The bootstrap analysis showed that most of the clades were poorly supported (Fig. 3). A few nodes that were well supported in the morphological data sets were *Roboastra* and *Nembrotha*. Within *Nembrotha* there were two sister clades. One clade including *Nembrotha lineolata*, *N. purpureolineata*, *N. chamberlaini*, *N. megalocera* and *N.* sp. 3 and one clade including*Nembrotha nigerrima*, *N. guttata*, *N. cristata*, *N. livingstonei*, *N. mullineri*, *N. milleri* and *N.* sp. 4. The relationships within each clade remained unclear. *Roboastra* was monophyletic with *R. gracilis* as sister species to the clade containing *R. luteolineata*, *R. europaea* and *R. tigris* + *R. leonis* + *R. caboverdensis*.

None of the main trees supported the current genus *Tambja* (Burn, 1962). The clade containing *T. limaciformis* + *T.amakusana* was sister clade of that containing *T. abdere* + *T. blacki* and *Nembrotha*, with *T. abdere* + *T. blacki* in a basal position to *Nembrotha*. In addition, the genus *Roboastra* was included within the remaining species of *Tambja*, thus making *Tambja* a polyphyletic assemblage.

3.4. Combined analysis (morphological and molecular data)

The strict consensus of all most parsimonious trees produced by the combined morphological and molecular data is shown in Fig. 4 (numbers above branches indicate bootstrap values). The one most parsimonious tree required 4115 steps (CI = 0.28; RI = 0.7). This tree, where all samples of Nembrothinae were included, indicated that the subfamily Nembrothinae was monophyletic. It split in four monophyletic clades: (1) one clade including *T. amakusana* and *T. limaciformis* (bs 99), (2) one including *T. abdere* and *T. blacki* (bs 89) (also recovered by the COI and morphology analyses), (3) another including *Nembrotha* species (bs 100) (recovered by all the analytical methods) and, (4) a group including all the remaining species of *Tambja* and *Roboastra* (bs 57). In the combined morphological and molecular analysis, the genus *Roboastra* was not monophyletic due to the presence of *Tambja tentaculata* (bs 70) within *Roboastra*. The monophyly of the genus *Tambja* was also rejected by the combined morphological and molecular analysis.

4. Discussion

The present study is the first attempt to elucidate the phylogenetic relationships among worldwide sea slugs of the subfamily Nembrothinae, an important taxon to understand trophic evolution, evolution of color patterns and vicariant biogeographic distributions. This study also provides an insight to the phylogeny of Doridoidea based on both morphological and molecular data. Regarding cryptobranch and "phanerobranch" dorids, our molecular data show the non-monophyly of these groups while morphological data support them as monophyletic groups. These results are in agreement with other published molecular (Thollesson, 1999b, 2000; Wollscheid and Wägele, 1999; Wollscheid-Lengeling et al., 2001; Vallés, 2002; Valdés and Templado, 2002; Grande et al., 2004) and morphological phylogenies (Wägele and Willan, 2000; Vallés, 2002; Fahey and Gosliner, 2004). Moreover, our study shows that *Ancula gibbosa* is more closely related to cryptobranchs than with "phanerobranch" dorids. So far, this result is also in agreement with some phylogenetic studies (Thollesson, 1999b, 2000; Vallés, 2002; Grande et al., 2004). Thus, we consider that in order to recover the monophyly of the traditional "Cryptobranchia", *Ancula gibbosa* should be included within the latter group. In that case, the diagnosis of Cryptobranchia would need to be revised. However, we also consider that more taxa should be included for further discussions. The monophyly of the family Polyceridae was also rejected by our molecular analyses.

Regarding Nembrothinae, the phylogenetic trees inferred from morphological, molecular and combined morphological and molecular characters mainly support identical relationships between genera and species. Further, almost all our phylogenetic reconstructions clearly support that the subfamily Nembrothinae is monophyletic and the splitting of it into several monophyletic clades. However, the position and the phylogenetic relationships of some taxa were not fully resolved.

Our results show that the most common morphological character used in the taxonomy of the subfamily Nembrothinae (e.g., presence of denticles on the upper side of the rachidian tooth) shows homoplasy according to our molecular and morphological reconstruction. On the other hand, some other characters, such as the differentiation and placement of the prostate, the vaginal gland, the vagina and the penial spines are phylogenetically informative and the results are congruent in both molecular and morphological analyses.

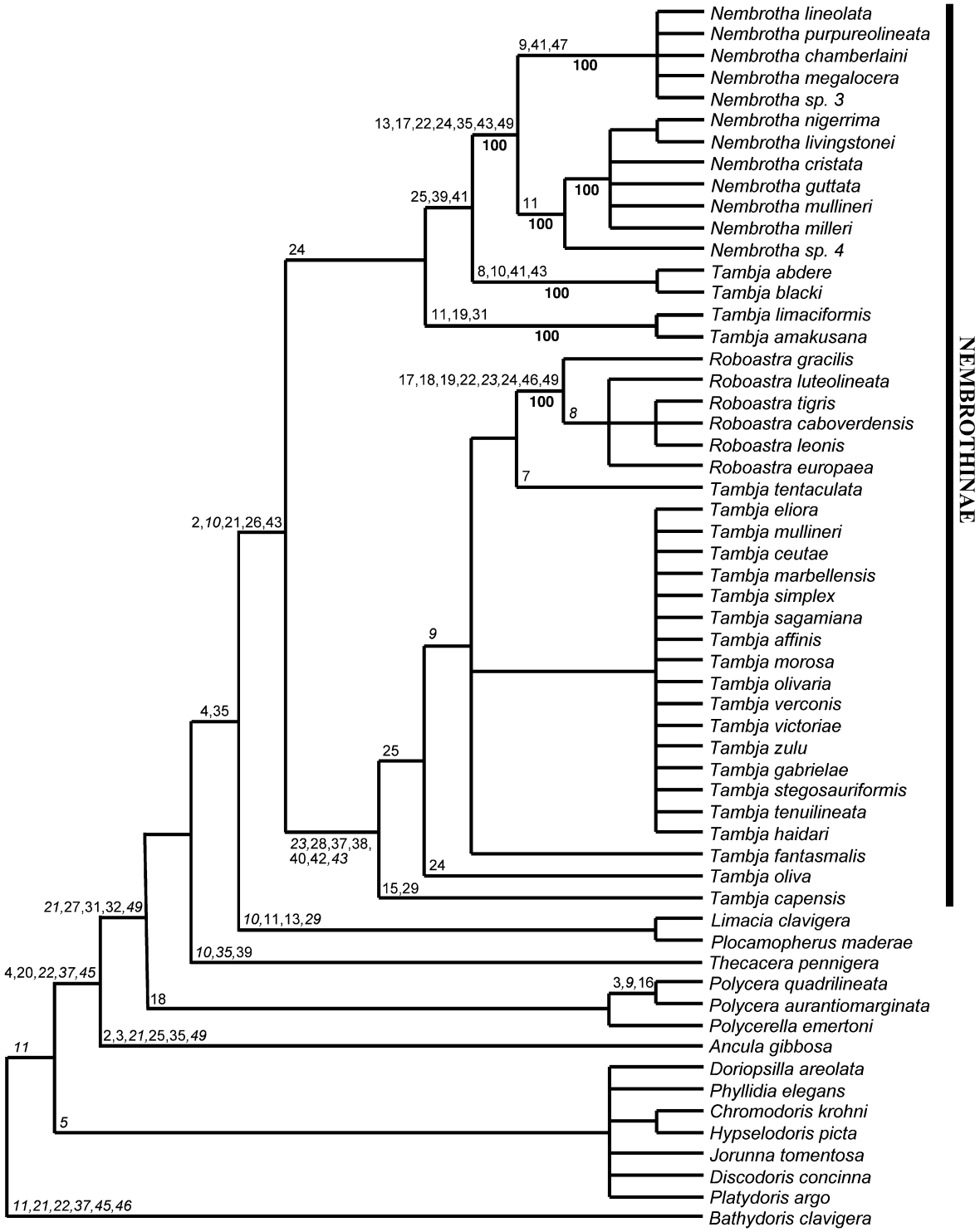

Fig. 3. Bootstrap consensus tree based on morphological characters showing character tracing. Numbers above branches refer to characters listed in Appendix A. Characters printed in italic face presented at least one instance of reversal. Numbers in bold refer bootstrap values.

Combining all the recovered data, the situation for each genus within Nembrothinae could be view as detailed below.

4.1. *Tambja*

Tambja's monophyly was not supported by morphological or molecular studies (Pola et al., 2006a), even when taxon sampling was improved (Pola et al., 2006c). Likewise, in the present study, molecular data alone or combined with morphological characters did not support the monophyly of *Tambja*. All our phylogenetic analyses based on molecular data revealed that *Tambja* clustered as six monophyletic clades with strong support: (1) *T. amakusana* + *T. limaciformis*, (2) *T. abdere* + *T. blacki* (when *T.*

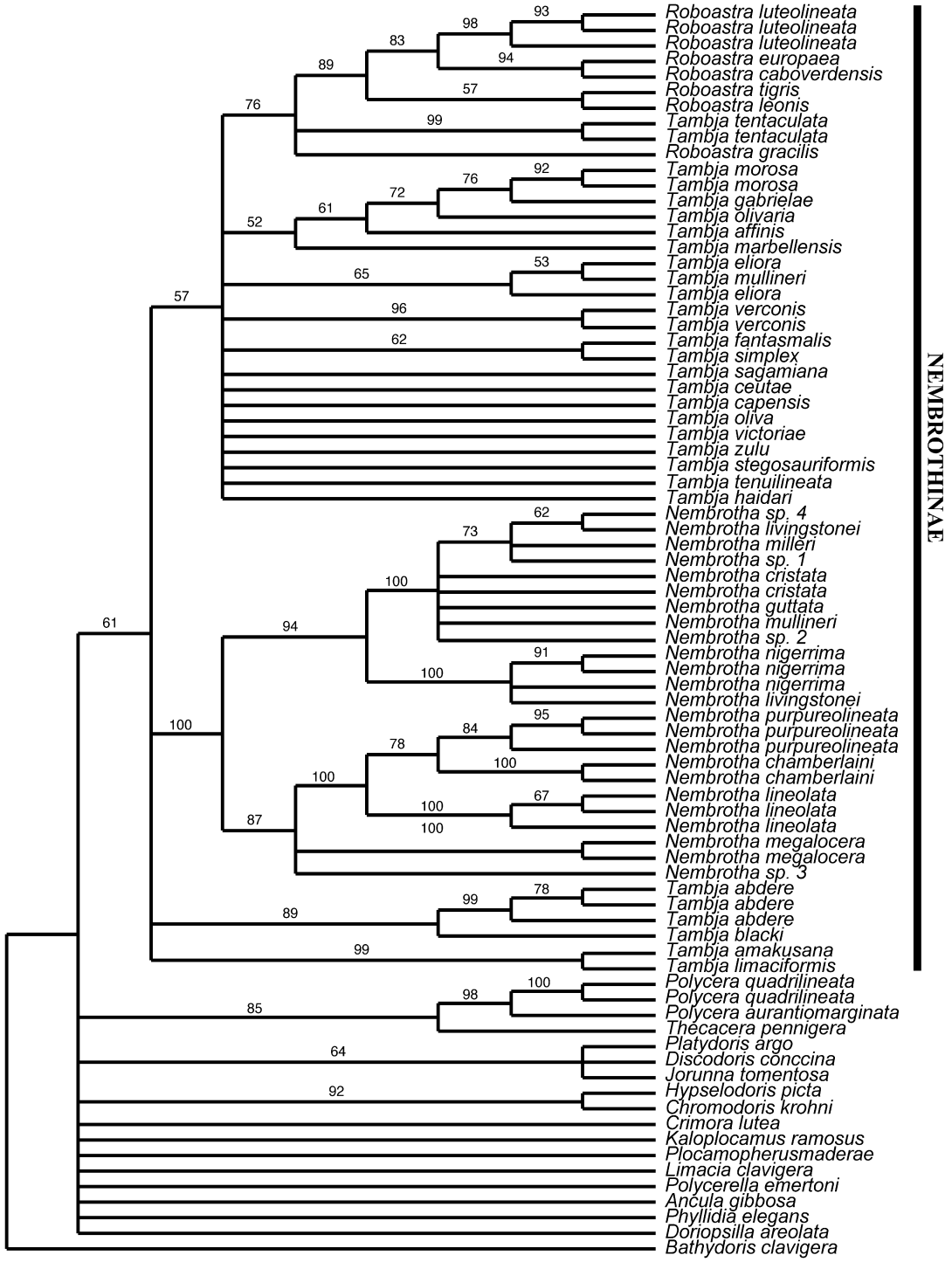

Fig. 4. Phylogenetic hypothesis based on "Total evidence" represented by a strict consensus maximum parsimony tree. Numbers in branches indicate bootstrap values.

blacki is included), (3) *T. capensis*, (4) *T. fantasmalis* + *T. simplex*, (5) *Roboastra* + *T. tentaculata* and, (6) the remaning species of *Tambja*. Supports for most of the basal nodes were not high and the relationships among the different clades were subjected to change by using different molecular data sets.

Tambja limaciformis resembles *T. amakusana* in its small size and in several internal features (Pola et al., 2006c). *Tambja abdere* from east Pacific and *T. blacki* from Australia and Papua New Guinea formed a well-supported monophyletic clade when *T. blacki* was included. *T. abdere* shares with *T. blacki* a very similar reproductive system arrangement (Pola et al., 2006a,b). *Tambja capensis* is the single species described from temperate southern Africa. In all our phylogenetic analyses this species was recovered in an independent clade. A recent detailed description of

this species is given by Pola et al. (2006c). The clade that includes *T. fantasmalis* and *T. simplex*, both from Cape Verde Islands, was supported by high posterior probability and 100% bootstrap support in all the molecular analysis. However, this strong relationship was not recovered by the morphological data (Fig. 3). The clade containing *Roboastra* + *T. tentaculata* was recognized in most of the analyses. In the combined molecular analysis (Fig. 4), this strongly supported clade was sister to the clade containing *T. fantasmalis* and *T. simplex*. However, this latter relationship was not supported. Internal relationships among these clades were not clearly established by analysis of the 16S sequence data. Finally, a clade including the major number of *Tambja* species was recovered by all the molecular phylogenetic analyses with high support. Some subgroups were strongly supported but, in general, the phylogenetic relationships within this clade were not fully established. Thus, *T. morosa*, *T. olivaria*, *T. gabrielae* and *T affinis* are closely related species and are also closely related to *T. verconis* and *T. sagamiana*. All these species share two morphological synapomorphies: a very high density of penial spines and the presence of irregular spots scattered on the notum. Moreover, they are all from the Indo-Pacific.

A Shimodaira–Hasegawa parametric test (Shimodaira and Hasegawa, 1999) comparing the ML topology with the ML topology obtained (by constraining all the samples of *Tambja* to form a monophyletic group indicated that the topologies were significantly different. Therefore, the monophyly of *Tambja* was statistically rejected based on our sampling.

Burn (1962, 1967) proposed the generic diagnosis based on morphological characters. Recently, Pola et al. (2006b,c) presented a review of the systematics of *Tambja* and found that several species did not perfectly match Burn's diagnosis. Additionally, a morphological character that had been considered determinant in taxonomic keys did not reveal its phylogenetic value, i.e, rachidian tooth without denticles with notched or smooth upper margin.

4.2. Roboastra

Roboastra is the least speciose genus with only six species. Unfortunately, *R. leonis* could not be sequenced for either of the two genes used (Table 2). To consider this group as monophyletic, *Tambja tentaculata* should be included in the genus. Morphological and combined morphological and molecular data showed *T. tentaculata* as sister species to the clade containing *Roboastra* species or included within *Roboastra* (Figs. 2–4). The relationship between *T. tentaculata* and *Roboastra* species was supported by the presence of a pair of well-developed, grooved, dorsolateral oral tentacles. Nevertheless, the presence of a thick labial cuticle, its radula with a notched, rectangular rachidian tooth without denticles and the absence of well developed lateral pouches led us to include this species within the genus *Tambja* (Pola et al., 2005b).

Within the cluster *Roboastra* + *T. tentaculata*, *R. gracilis* appeared, in most of the analyses, in a basal polytomy with *T. tentaculata* (Figs. 2 and 4). This species presents several marked morphological differences with respect to the remaining *Roboastra* spp (Pola et al., 2005a). The molecular phylogenetic results also highly supported *R. europaea* being closely related to *R. caboverdensis*. In the overall molecular analyses, we also found that *R. luteolineata* appeared as sister taxon to *R. europaea* + *R. caboverdensis* with *R. tigris* in a basal position to the latter clade. However, these relationships are different from the ones obtained with the morphological characters (Pola et al., 2005a). Thus, the singularity of this group is always supported but further research may clarify these potential relationships.

4.3. Nembrotha

Nembrotha was recognized in all the analyses as a monophyletic group. Support was always high, confirming the separation made by Burn (1967) of *Nembrotha* from the *Roboastra* and *Tambja*, based on morphological characters. The synapomorphies that characterize the genus *Nembrotha* are: a weak labial cuticle with strong internal edge and a well differentiated prostate spreading entirely over the bursa copulatix. Moreover, all species of this clade feed on tunicates. There are two well-differentiated subgroups within the *Nembrotha* clade. This subdivision corresponds to four marked morphological differences: the "spotted" versus "lined" external pattern on the notum, the types of penial spines, the position of the penial spines on the penis and the shape of the vagina. The clade containing the "lined" species (*N. chamberlaini*, *N. purpureolineata*, *N. lineolata*, *N.* sp. 3 and *N. megalocera*) has only one type of hooked penial spines, lack penial spines at the base of the penis and has a very convoluted vagina while the clade containing the "spotted" species (*N. nigerrima*, *N. livingstonei*, *N. cristata*, *N. guttata*, *N. mullineri*, *N. milleri*, *N.* sp. 4, *N.* sp. 1 and *N.* sp. 2) is characterized by the presence of two or three different types of penial spines, starting at the base of the penis, and the shape of the vagina is straight. Two subgroups within the "spotted" clade were strongly supported but, in general, the phylogenetic relationships within the subgroups were not fully established. Within the "lined" clade, the phylogenetic relationships based on morphological characters observed here did not resolve the relationships among the "lined" *Nembrotha* species. However, molecular data clearly distinguished between different species. Thus, *N. megalocera* and *N.* sp. 3 always appeared in a basal polytomy with the clade including *N. purpureolineata*, *N. lineolata* and *N. chamberlaini*. In both combined analyses we found that *N. purpureolineata* is more closely related to *N. chamberlaini* than to other species and *N. lineolata* appeared basal to them, while the COI and 16S sequences showed *N. chamberlaini* as sister species to *N. lineolata* and *N. purpureolineata* in a basal position. There are several possible explanations for the incomplete resolution of internal relationships within the

"spotted" *Nembrotha*. Either morphological characters within this group show convergence or the genes screened here were inadequate for analyzing this group. Considering the number of informative positions and the lack of saturation, this latter possibility seems not to be the case. Alternatively, the polytomies detected could also be explained by rapid radiation. Vicariant events are considered the triggers for speciation. Nevertheless, sudden changes in habitat conditions followed by isolation, or the colonization of new ecological niches, could lead to the splitting of a previous more or less widely distributed taxon into several new taxa. If these changes or isolation phenomena occur sufficiently rapidly, there is no genetic signal reflecting the phylogenetic relationships. Further research with more genes may clarify these potential relationships.

4.4. Taxonomic implications

The morphological and molecular analyses undertaken allowed us to suggest that *Tambja* is not a monophyletic group. Thus, the traditional classification of the subfamily Nembrothinae needs to be revised. Several systematic alternatives are proposed in Fig. 5 but further studies need to be done for taxonomic decisions.

The alternatives 2 and 3 do not seem to add any information that is not already contained in the phylogeny and add unnecessary names to the nomenclature. Thus, the first alternative would appear the simplest solution that preserves the monophyly and does not erect a nomenclature that would be unnecessarily complicated.

Alternative 1. Nembrothinae Burn, 1967
Nembrotha Bergh, 1877
Roboastra s.l. (new definition)

Alternative 2. Nembrothinae Burn, 1967
Nembrotha Bergh, 1877
Roboastra Subgenus *Roboastra* s.s.
Roboastra Subgenus*Tambja* s.s.
Roboastra Subgenus A
Roboastra Subgenus B
Roboastra Subgenus C
Roboastra Subgenus D

Alternative 3. Nembrothinae Burn, 1967
Nembrotha Bergh, 1877
Roboastra Bergh, 1877
*Tambja*s.s. Burn, 1962
Genus A'
Genus B'
Genus C'
Genus D'

Fig. 5. Proposed alternatives for the classification of the subfamily Nembrothinae based on monophyletic groups. In alternative 1, all *Tambja* and *Roboastra* taxa are included within a single monophyletic taxon, *Roboastra* s.l. In alternative 2, six subgenera are erected, one including all species of *Roboastra* s.s., one including all species of *Tambja* s.s. and for new subgenera corresponding to the monophyletic clades: A = *T. fantasmalis* + *T. simplex*; B = *T. amakusana* + *T. limaciformis*; C = *T. abdere* + *T. blacki*; D = *T. capensis*. In alternative 3, the traditional genera are maintained, plus four new genera (A′, B′, C′, D′).

Nevertheless, all classifications proposed here will need further refinement and improvement. For instance, further analyses including all the species described for each clade and adding more genes that help to elucidate the basal relationships, are necessary to clarify the relationship between these taxa.

Thus, for practical reason, we propose to mantaine the use of the name "*Tambja*" s. l. Burn, 1962 until further studies can clarify this situation.

Regarding the monophyletic group *Nembrotha* the external morphology within *Nembrotha* is highly variable and we have not been able to examine all different variable forms and intermediate states of each species, nor even include all these forms in the molecular analyses. As a result we have preferred to maintain a conservative point of view and thus maintain most of the nominal species of the genus, as this highlights differences rather than lumping taxa whose variability is not fully understood. Further anatomical and molecular studies, as well as studies of populations and biogeographical studies, need to be done in order to know if some of *Nembrotha* species could be just varieties of other species as suggested by our morphological (Pola, Cervera and Gosliner, submitted for publication) and molecular analyses.

Finally, the trees obtained from the phylogenetic analyses indicate that the status of the traditional family Polyceridae has to be revised. Thus, the subfamily Nembrothinae is clearly monophyletic but the monophyly of Polyceridae is not supported by our analyses. Wägele and Willan (2000) and Thollesson (1999b, 2000) also found this latter result.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.02.003.

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