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The phylogeny of Nudibranchia (Opisthobranchia, Gastropoda, Mollusca) reconstructed by three molecular markers

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Abstract

The phylogeny of the Nudibranchia and its major constituent taxa is investigated by comparing the complete sequences of the 18S rDNA of 54 species, a part of the 16S rDNA of 38 species and part of cytochrome *c* oxidase I (*cox1*) of 45 species. These datasets are analyzed individually and in combination for the subset of taxa where information on all three markers is available. The results are compared to published cladistic analyses based on morphological data. The monophyly of the Nudibranchia and the monophyly of its two major groups, the Anthobranchia/Doridoidea and Cladobranchia, is confirmed. Incongruencies between the molecular and morphological data is discussed, as well as incongruencies between the three molecular markers.

Key words: Nudibranchia, 18S rDNA, 16S rDNA, *cox1*, molecular phylogeny

Introduction

The Nudibranchia, a subgroup of the Opisthobranchia (Gastropoda), are often called butterflies of the ocean because of their body forms and attractive colours. They live exclusively in marine habitats from the intertidal to the deep sea, and have worldwide distribution from the polar regions to the tropics. Their shell-less bodies show manifold forms, and they have adopted diverse foraging strategies. They often exploit prey that is hardly used by other marine invertebrates, and some species have evolved the capability to incorporate and use the defence systems of their prey, e.g. the toxic chemicals of sponges, or the cnidocysts of cnidarians. Others produce defensive systems de novo (chemicals and/or spicules).

Opisthobranchia and Pulmonata usually have been united under the name Euthyneura (Boettger 1955), one of the major branches of the Gastropoda. Traditionally, the other major branch has been the Prosobranchia, but recent investigations have shown this group to be paraphyletic and demonstrated close relations of some prosobranchs, Valvatoidea and Architectonicoidea, with the Euthyneura, resulting in a new grouping, the Heterobranchia (Haszprunar 1985, 1988). Furthermore, both the monophyly of the Pulmonata and that of the Opisthobranchia remain uncertain (e.g. Tillier et al. 1994, Mikkelsen 1996, Ponder & Lindberg 1997, Winnepenninckx et al. 1998). Figure 1 shows our present knowledge of relationships among the major groups within the Opisthobranchia, based on cladograms published by Mikkelsen (1996) and Wägele & Willan (2000).

Nudibranchia has been viewed as monophyletic by many authors (Boettger 1955, Tardy 1970, Schmekel 1985), although some alternatively suggested that they are paraphyletic (Bergh 1892, Pelseneer 1893–1894, Minichev 1970). The most recent, comprehensive cladistic studies on the phylogeny of the Nudibranchia (Wägele 1997, Wägele & Willan 2000) proposed a number of synapomorphies in favour of nudibranch monophyly. This was corroborated by Wollscheid & Wägele

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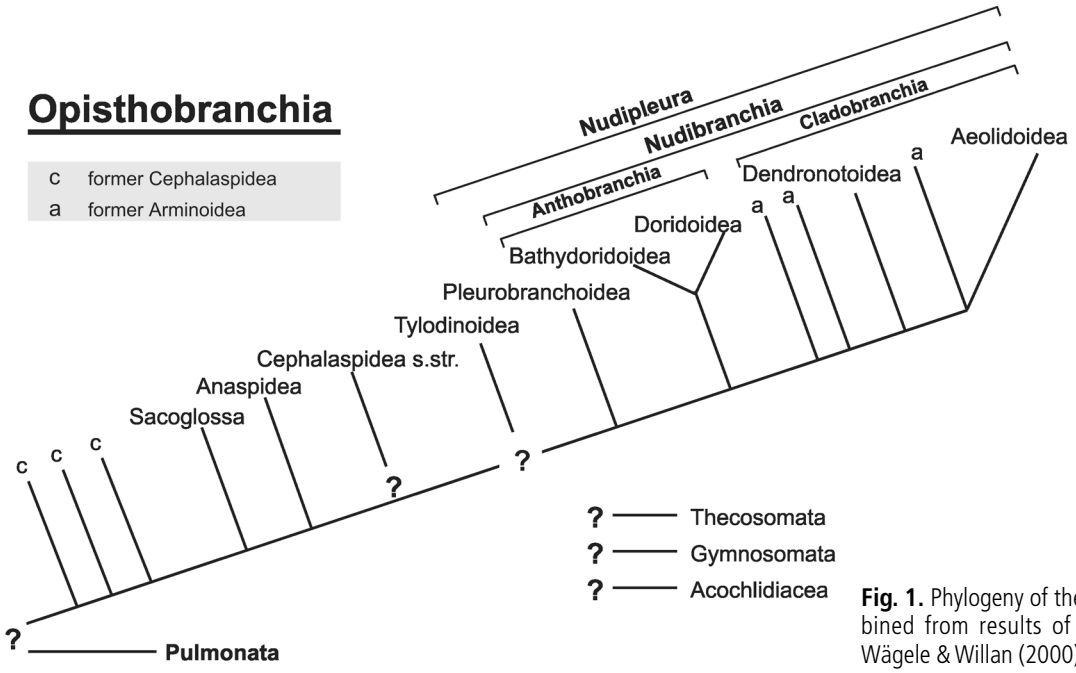


Fig. 1. Phylogeny of the Opisthobranchia, combined from results of Mikkelsen (1996) and Wägele & Willan (2000).

(1999) through a comparison of the complete 18S rDNA sequences of 53 gastropods, including 19 nudibranch taxa. However, Thollesson (1999a) concluded that Nudibranchia is paraphyletic based on his comparison of part (approximately 480 bp) of the 16S rDNA of nearly 30 gastropods.

Within the Nudibranchia (Fig. 1), two major groups (Cladobranchia and Anthobranchia) have been recognized for nearly 200 years (Férussac 1822). Within the Cladobranchia Odhner (1934) advanced three major taxa, the Dendronotoidea, Arminoidea and Aeolidoidea. Within the Anthobranchia he recognized only the single order Doridoidea. Wägele (1989) split the Doridoidea into two sister taxa, forming the new order Bathydoridoidea (Fig. 1). Wägele & Willan (2000) assumed paraphyly for the Arminoidea.

The molecular data presented by Wollscheid & Wägele (1999) and Thollesson (1999a) support monophyly for each of the two clades Cladobranchia and Anthobranchia, but within these taxa, the analyses are inconsistent regarding monophyly versus paraphyly of the Aeolidoidea, Dendronotoidea, and Arminoidea.

These conflicting hypotheses on relationships concerning the Nudibranchia and its subordinate taxa are addressed in the present study by including a larger number of sequences of nudibranch and outgroup species. Complete sequences of 18S (SSU) rDNA from the nucleus and 16S (LSU) rDNA and *cox1* from the mitochondrial genome of 38 to 54 different opisthobranch species have been determined and compared. Comparison of these nucleotides and inferred amino acid sequences are used to address the monophyly of Nudibranchia and the derivation of its subordinate taxa. Due to lack of more information on other opisthobranch groups, the position of the Nudibranchia within the Opisthobranchia, as well as the monophyly or paraphyly of other opisthobranch taxa and their position, can not be clarified yet. This is the largest molecular dataset to date for addressing questions of phylogenetic relationship of nudibranchs.

Material and methods

The complete sequences of 18S rDNA were determined for 54 species. Three additional sequences were taken from GenBank (Littorinoidea: *Littorina littorea*, X91970, *Littorina obtusata*, X94274 and *Aplysia* spec., X94268). The studied taxa, along with their locations of collection and the GenBank accession numbers for their sequences, are shown in Table 1. Alignments can be ordered from the corresponding author.

The 18S rDNA fragments were amplified using primers matching conserved regions (18A1: 5'CCT ACT CTG GTT GAT CCT GCC AGT; 1800: 5'TAA TGA TCC TTC CGC AGG TT) using PCR (38 cycles of 30 s at 94 °C, 50 s at 52.5 °C, 2.5 min at 72 °C). Amplifications were made from whole genomic preparations. The PCR product was, at the beginning of this project, cloned using a TA Cloning Kit (Invitrogen) and sequenced with fluorescent labelled primers using a Thermo Sequenase cycle sequencing kit (Amersham). After establishing a direct sequencing protocol, 18S rDNA fragments were later sequenced directly. For the

Table 1. Species investigated, with collection sites and GenBank accession numbers for sequences from three genes

Taxon	Collection site	18S rDNA	16S rDNA	*cox1*
PULMONATA				
Cepaea nemoralis Linné, 1758	Germany, Bielefeld	AJ224921	AF249259	
CEPHALASPIDEA s. l.				
Haminoea cymbalum (Quoy & Gaimard, 1935)	Australia, Great Barrier Reef	AF249221	AF249258	
Smaragdinella spec.	Egypt, Red Sea	AJ224789	AF249257	AF249806
SACOGLOSSA				
Elysia timida Risso, 1818	Spain, Mediterranean Sea			AF249818
Limapontia nigra (Müller, 1733)	North Sea	AJ224920		
Thuridilla bayeri Marcus, 1965	Australia, Great Barrier Reef	AF249220		
Thuridilla hopei (Verany, 1853)	Australia, Great Barrier Reef			AF249810
Thuridilla ratna Marcus, 1965	Australia, Great Barrier Reef		AF249256	
ANASPIDEA				
Aplysia depilans Bohatsch, 1761	Normandy, NE Atlantic	AJ224918		AF249824
Aplysia extraordinaria Allan, 1932	Australia, Great Barrier Reef	AF249193	AF249255	AF249823
Aplysia parvula Mörch, 1863	Spain, Mediterranean Sea			AF249822
Aplysia punctata Cuvier, 1803	Helgoland, North Sea	AJ224919	AF249253	
Aplysia spec.	Spain, NE Atlantic	AF249192	AF249254	
TYLODINOIDEA				
Tylodina perversa (Gmelin, 1790)	Spain, Mediterranean Sea			AF249809
PLEUROBRANCHOIDEA				
Bathyberthella antarctica (Willan & Bertsch, 1987)	Antarctica, Weddell Sea	AF249219		
Berthellina citrina (Rüppel & Leuckart, 1828)	Spain, Mediterranean Sea			AF249785
Euselenops luniceps (Cuvier, 1817)	Australia, Great Barrier Reef	AF249218		
NUDIBRANCHIA				
BATHYDORIDOIDEA				
Bathydoris clavigera Thiele, 1912	Antarctica, Weddell Sea		AF249222	AF249808
DORIDOIDEA				
Acanthodoris pilosa (Müller, 1776)	Helgoland, North Sea	AJ224770	AF249236	
Adalaria proxima Alder & Hancock, 1854			AF249225	
Archidoris pseudoargus (Rapp, 1827)	Helgoland, North Sea	AF249217	AF249224	
Austrodoris kerguelenensis (Bergh, 1884)	Antarctica, Weddell Sea	AJ224771	AF249233 AF249780	AF249234
Cadlina luteomarginata (MacFarland, 1966)	USA, North Atlantic	AJ224772	AF249231	AF249803
Chromodoris krohni (Verany, 1846)	Spain, NE Atlantic	AJ224774	AF249239	AF249805
Chromodoris kuiteri (Rudman, 1982)	Australia, Great Barrier Reef	AF249214	AF249240	AF249804
Chromodoris luteorosea (Rapp, 1827)	Spain, Mediterranean Sea			AF249815
Chromodoris quadricolor (Rüppel & Leuckart, 1828)	Egypt, Red Sea	AJ224773	AF249241	AF249802
Crimora papillata Alder & Hancock, 1862	Spain, Mediterranean Sea			AF249821
Dendrodoris fumata (Rüppel & Leuckart, 1828)	Australia, Great Barrier Reef	AF249216		AF249799
Dendrodoris nigra (Stimpson, 1855)	Australia, Great Barrier Reef	AF249215	AF249242	AF249795
Diaphorodoris luteocincta (Sars, 1870)	Spain, NE Atlantic	AJ224775	AF249230	AF249796
Diaphorodoris papillata Portmann & Sandmeier, 1960	Spain, Mediterranean Sea			AF249819
Discodoris atromaculata Bergh, 1880	Turkey, Mediterranean Sea			AF249784
Discodoris concinna (Alder & Hancock, 1864)	Australia, Great Barrier Reef Dominican Republic, Caribbean Sea	AF249213 AF249228	AJ224781	AF249801
Doriopsis granulosa Pease, 1860	Australia, Great Barrier Reef	AF249212	AF249223	AF249798
Glossodoris atromarginata (Cuvier, 1804)	Australia, Great Barrier Reef	AF249211		AF249789
Goniodoris nodosa (Montagu, 1808)	Spain, NE Atlantic	AJ224783	AF249226	AF249788
Hypselodoris elegans (Cantraine, 1834)	Spain, NE Atlantic	AJ224779	AF249238	AF249787
Hypselodoris villafranca (Risso, 1818)	Spain, NE Atlantic	AJ224780	AF249237	
Jorunna tomentosa (Cuvier, 1804)	Helgoland, North Sea	AF249210		
Limacia clavigera (Müller, 1776)	Spain, NE Atlantic	AJ224778		
Onchidoris bilamellata (Linné, 1767)	Helgoland, North Sea	AJ224776	AF249235	
Phyllidia coelestis Bergh, 1905	Australia, Great Barrier Reef	AF249209		
Phyllidiella pustulosa (Cuvier, 1804)	Australia, Great Barrier Reef	AF249208	AF249232	
Platydoris argo (Quoy & Gaimard, 1832)	Spain, Mediterranean Sea			AF249811

Table 1. Continued

Taxon	Collection site	18S rDNA	16S rDNA	*cox1*
Plocamopherus ceylonicus (Kelaart, 1885)	Australia, Great Barrier Reef	AF249207		
Polycera quadrilineata (Müller, 1776)	Kattegat, North Sea	AJ224777	AF249229	
Triopha catalinae (Cooper, 1863)	USA, North Atlantic	AJ224782	AF249227	
DENDRONOTOIDEA				
Dendronotus dalli Bergh, 1879	USA, North Atlantic		AF249252	AF249800
Dendronotus frondosus (Ascanius, 1774)	Kattegat, North Sea	AF249206	AF249251	
Doto coronata (Gmelin, 1791)	Kattegat, North Sea	AF249203		AF249794
Doto floridicula Simroth, 1888	Spain, Mediterranean Sea			AF249820
Doto eireana Lemche, 1976	Spain, NE Atlantic	AF249204	AF249248	
Doto koenneckeri Lemche, 1976	Spain, NE Atlantic	AF249205	AF249249	AF249797
Doto pinnatifida (Montagu, 1804)	Spain, NE Atlantic	AF249202	AF249250	AF249793
Marionia blainvillea Risso, 1828	Spain, Mediterranean Sea			AF249812
Melibe leonina (Gould, 1852)	USA, North Atlantic	AJ224784		
Tritoniella belli Eliot, 1907	Antarctica, Weddell Sea	AF249201		
Tritonia nilsodhneri Marcus, 1983	Spain, NE Atlantic	AF249200		
Tritonia plebeia Johnston, 1828	Helgoland, North Sea	AJ224785		
"ARMINOIDEA"				
Armina loveni (Bergh, 1860)	Kattegat, North Sea	AF249196	AF249243	AF249781
Dermatobranchus semistriatus Baba, 1949	Australia, Great Barrier Reef	AF249195	AF249244	
Janolus cristatus delle Chiaje, 1841	Osterschelde, North Sea	AF249194		AF249813
AEOLIDOIDEA				
Cratena peregrina Gmelin, 1791	Spain, Mediterranean Sea			AF249786
Cuthona caeruela (Montagu, 1804)	Kattegat, North Sea	AF249199		AF249807
Eubranchus exiguus (Alder & Hancock, 1848)	Helgoland, North Sea	AJ224787	AF249246	AF249792
Eubranchus spec.	Spain, NE Atlantic	AJ224786		AF249791
Facelina punctata Alder & Hancock, 1845	Spain, Mediterranean Sea			AF249816
Flabellina affinis (Gmelin, 1791)	Spain, Mediterranean Sea			AF249783
Flabellina ischitana Hirano & Thompson, 1990	Spain, Mediterranean Sea			AF249814
Flabellina pedata (Montagu, 1814)	Helgoland, North Sea	AJ224788	AF249247	AF249817
Flabellina verrucosa (Sars, 1829)	USA, NW Atlantic	AF249198	AF249245	AF249790
Godiva banyulensis (Garcia & Garcia, 1985)	Spain, Mediterranean Sea			AF249782
Tergipes tergipes (Forskal, 1775)	Kattegat, North Sea	AF249197		
GENBANK:				
ANASPIDEA				
Aplysia spec.		X94268		
„PROSOBRANCHIA"				
Littorina littorea (Linné, 1758)		X91970		
Littorina obtusata (Linné, 1758)		X94274		

18S rDNA, only one clone/DNA fragment was sequenced for each species. Further details of DNA extraction, amplification and sequencing are as previously described (Wollscheid & Wägele 1999). Additionally, fragments of two mitochondrial genes were amplified using PCR conditions similar to those above. A 500 bp fragment near the 3' end of the mitochondrial 16S rDNA was amplified from 38 species using primers 16Sbrh and 16Sarl (Simon et al. 1994). A 597 bp coding region near the 5' terminus of *cox1* was amplified from 45 species using primers LCO1490 (GGT CAA CAA ATC ATA AAG ATA TTG G) and HCO2198 (TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer et al. 1994). PCR products were purified by three cycles of ultrafiltration with Ultrafree spin columns (30,000 NMWL; Millipore), and sequenced directly using a Dye Terminator cycle sequencing kit (Applied Biosystems).

With the exception of the Bathydoridoidea, for which no 18S rDNA sequence was analysed (due to lack of appropriate material), all five major groups of the Nudibranchia were sampled for all three genes.

The sequences were initially aligned using ClustalX (Multiple Alignment Mode) (Thompson et al. 1997), then these alignments were refined by hand (e.g., removing gaps incorporated in one position for all species by

ClustalX) using the computer program Genedoc (Nicholas & Nicholas 1997). The reading frame was preserved in the alignment of the *cox1* sequences.

The aligned sequences were subjected to phylogenetic analysis using Maximum Likelihood (ML) in PHYLIP (Felsenstein 1995), Neighbor Joining (NJ, Kimura 2-parameter model) as implemented in MEGA 1.01 (Kumar et al. 1993; options „Complete deletion of gaps" and „Pairwise deletion of gaps" were both tested) and Maximum Parsimony (MP) methods (PAUP 4.0; Swofford et al. 1996). For the MP analysis the heuristic search option (ACCTRAN or alternatively DELTRAN) was used with the following settings: branch swapping: closest; nearest neighbour interchange or alternatively tree bisection reconnection; 50% majority-rule consensus tree. Bootstrap analyses contained 1000 replicates, gaps were treated as missing. ML analyses of the sequences were performed exclusively with DNAML (Phylip), with the following settings for DNA sequences: search for best tree, use empirical base frequencies, four categories of substitution rates (0.5, 1, 2 and 5; determined by statistical analyses of the sequences in MEGA). Due to the large data sets, the option of random input order of sequences was only chosen in very few analyses. Results of these analyses did not deviate from those with input of sequences by order. A parsimony analysis for protein sequences was performed by applying PROTPARS (PHYLIP) with the following settings for inferred amino acid sequences: use threshhold parsimony: no, analyse multiple data sets: no. Due to the lack of appropriate sequences and due to the large data set, it was not possible to use the same „prosobranch" outgroup for all genes. In the 18S rDNA analysis, representatives (*Littorina*) of the sister group of the Heterobranchia, the Caenogastropoda (s. Haszprunar 1988, Ponder & Lindberg 1997), were used to root the tree. For the more rapidly evolving 16S rDNA, a more closely related outgroup species was selected, the pulmonate *Cepaea nemoralis*. Unfortunately, data on *Cepaea nemoralis* and/or members of the Caenogastropoda were not available for the *cox1* analyses, thus a species (*Smaragdinella* spec.*)* investigated here and belonging to the Cephalaspidea s. str. (Mikkelsen 1996) was used to root the tree. Finally, to avoid misinterpretations due to the differing outgroups, phylogenetic analyses of the Nudibranchia were also performed by including only opisthobranch taxa and using *Smaragdinella* spec. as the outgroup for rooting. Only those 19 species have been included in the combined analysis of the three markers, for which information on all markers was available.

Evolutionary rate variation was assessed using LINTRE (Takezaki et al. 1995) following the Wu & Li (1985) relative rate test.

Results

The alignment resulted in 2468 positions for the 18S rDNA, in 465 positions for the 16S rDNA, and in 597 positions (or 199 inferred amino acids) for the *cox1* gene. The overall base composition of the 18S rDNA genes was slightly more than half G+C, whereas the two mitochondrial genes have a compositional bias favouring A+T. The differences in base composition bias between species under consideration were not significant χ^2 tests: p = 0.000000 for 18S rDNA, p = 0.000137 for 16S rDNA, p = 0.000115 for *cox1*), thus compositional bias should not have interfered with the recovery of phylogenetic signal. Alignment for the combined analysis resulted in 2345 positions.

Unambiguous alignments were obtained for most portions of the three genes. However, several divergent domains, particularly in the 18S rDNA, showed regions of difficult alignment due to insertions in the taxa Nudibranchia and Pleurobranchoidea. Phylogenetic analyses were performed with and without these insertions, and the results were identical. Therefore, the insertions were not excluded from subsequent phylogenetic analyses.

The data set consisted of 1383 variable and 967 parsimony informative sites for the 18S rDNA gene, 289 variable and 233 parsimony informative sites for the 16S rDNA gene, 368 variable and 326 parsimony informative sites for the *cox1* gene, 112 variable and 85 parsimony informative sites for the *cox1* inferred amino acids, and 834 variable and 561 parsimony informative sites for the combined markers. The transition/transversion (TS/TV) ratio observed among species varied between 5 for closely related species and 0.5 between species from different higher taxa. ML analyses performed with different settings for TS/TV ratio yielded identical or congruent topologies; thus, only one ML tree each is given below for the 16S rDNA (Fig. 5) and *cox1* gene (Fig. 6).

The robustness of these results is supported by the high bootstrap values obtained in the MP analyses (included in Figs 3–4). Choosing *Smaragdinella* spec. as outgroup in the 18S and 16S analyses did not affect tree topologies.

The analyses of the 18S and 16S data sets, using all different phylogenetic methods with the different options and settings as mentioned above in Material and methods, support a monophyletic Nudibranchia clade (Figs 2–5). In the *cox1* analyses (DNA as well as amino acid sequences) the pleurobranchoid species *Berthellina citrina* is placed within the Doridoidea with a varying position, and thus renders the Nudibranchia paraphyletic (Fig. 6). The combined analysis renders the Nudibranchia monophyletic (Fig. 7). In the 18S analyses the two members of the Pleurobranchoidea form the sister taxon to the Nudibranchia. Both species investigated here are characterized by a long insertion between posi-

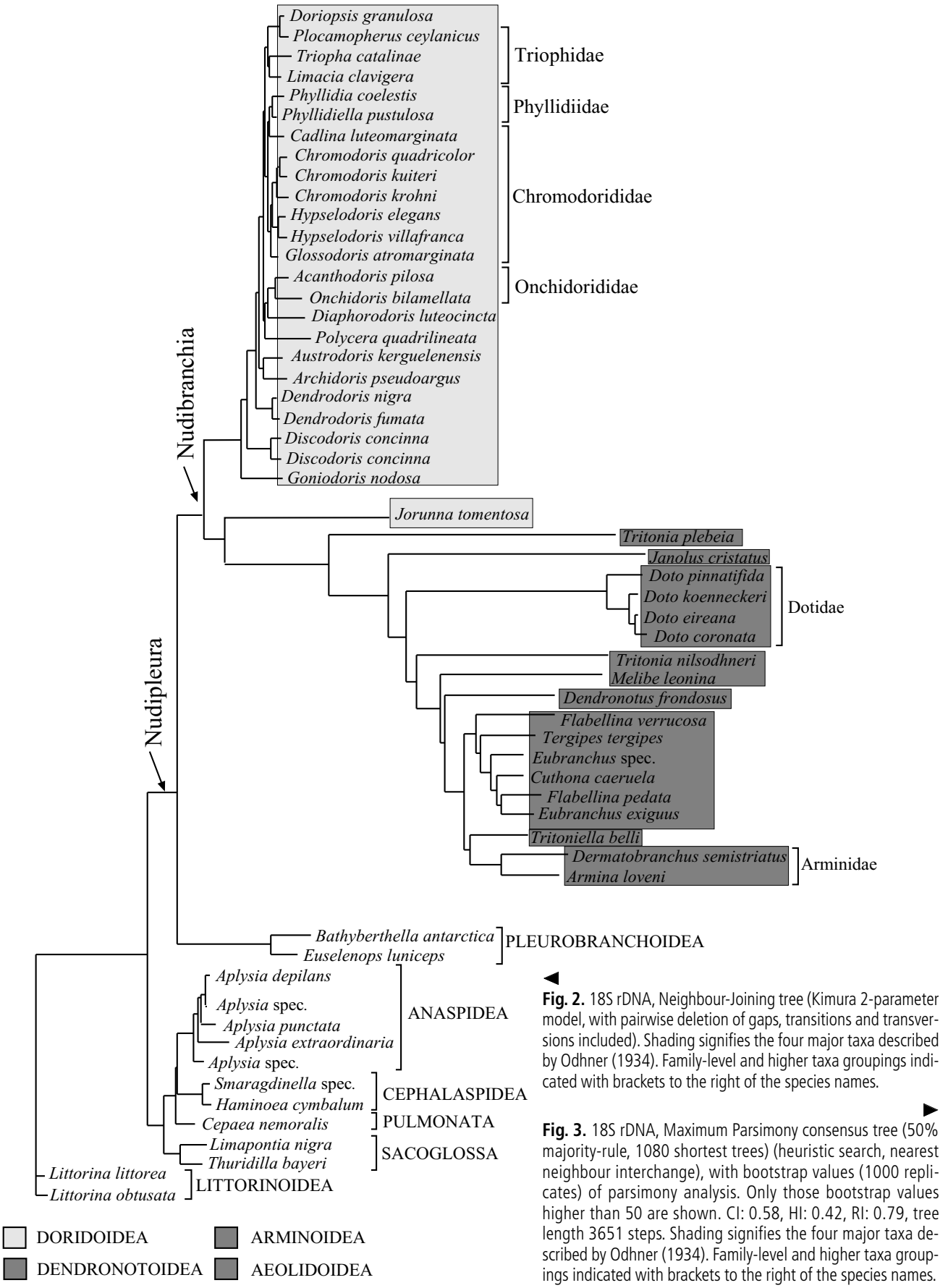
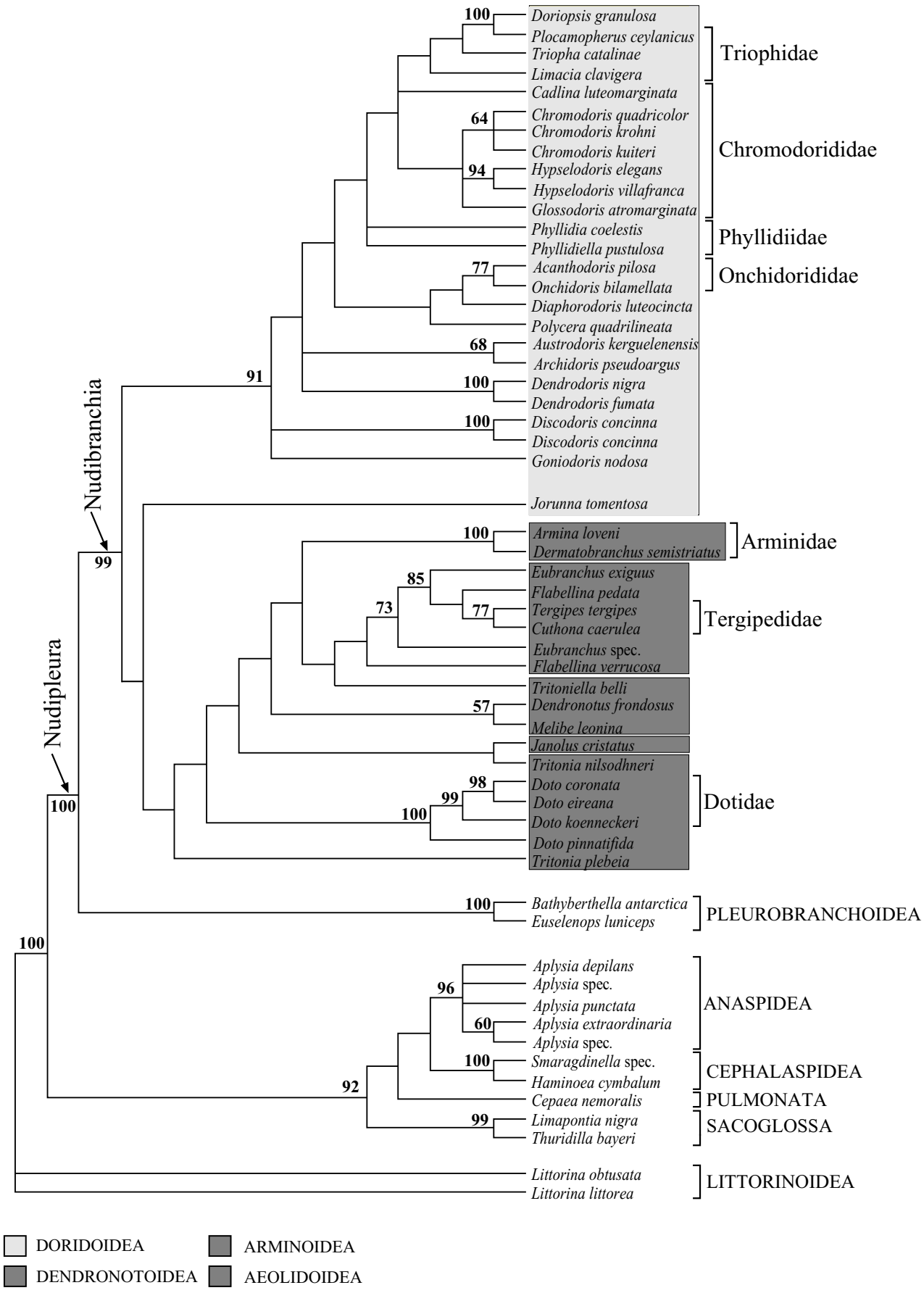


Fig. 2. 18S rDNA, Neighbour-Joining tree (Kimura 2-parameter model, with pairwise deletion of gaps, transitions and transversions included). Shading signifies the four major taxa described by Odhner (1934). Family-level and higher taxa groupings indicated with brackets to the right of the species names.

Fig. 3. 18S rDNA, Maximum Parsimony consensus tree (50% majority-rule, 1080 shortest trees) (heuristic search, nearest neighbour interchange), with bootstrap values (1000 replicates) of parsimony analysis. Only those bootstrap values higher than 50 are shown. CI: 0.58, HI: 0.42, RI: 0.79, tree length 3651 steps. Shading signifies the four major taxa described by Odhner (1934). Family-level and higher taxa groupings indicated with brackets to the right of the species names.


Doriopsis granulosa
Plocamopherus ceylanicus
Triopha catalinae
Limacia clavigera
Cadlina luteomarginata
Chromodoris quadricolor
Chromodoris krohni
Chromodoris kuiteri
Hypselodoris elegans
Hypselodoris villafranca
Glossodoris atromarginata
Phyllidia coelestis
Phyllidiella pustulosa
Acanthodoris pilosa
Onchidoris bilamellata
Diaphorodoris luteocincta
Polycera quadrilineata
Austrodoris kerguelenensis
Archidoris pseudoargus
Dendrodoris nigra
Dendrodoris fumata
Discodoris concinna
Discodoris concinna
Goniodoris nodosa
Jorunna tomentosa
Armina loveni
Dermatobranchus semistriatus
Eubranchus exiguus
Flabellina pedata
Tergipes tergipes
Cuthona caerulea
Eubranchus spec.
Flabellina verrucosa
Tritoniella belli
Dendronotus frondosus
Melibe leonina
Janolus cristatus
Tritonia nilsodhneri
Doto coronata
Doto eireana
Doto koenneckeri
Doto pinnatifida
Tritonia plebeia
Bathyberthella antarctica
Euselenops luniceps
Aplysia depilans
Aplysia spec.
Aplysia punctata
Aplysia extraordinaria
Aplysia spec.
Smaragdinella spec.
Haminoea cymbalum
Cepaea nemoralis
Limapontia nigra
Thuridilla bayeri
Littorina obtusata
Littorina littorea
Triophidae
Chromodorididae
Phyllidiidae
Onchidorididae
Arminidae
Tergipedidae
Dotidae
PLEUROBRANCHOIDEA
ANASPIDEA
CEPHALASPIDEA
PULMONATA
SACOGLOSSA
LITTORINOIDEA
Nudibranchia
Nudipleura
DORIDOIDEA
ARMINOIDEA
DENDRONOTOIDEA
AEOLIDOIDEA

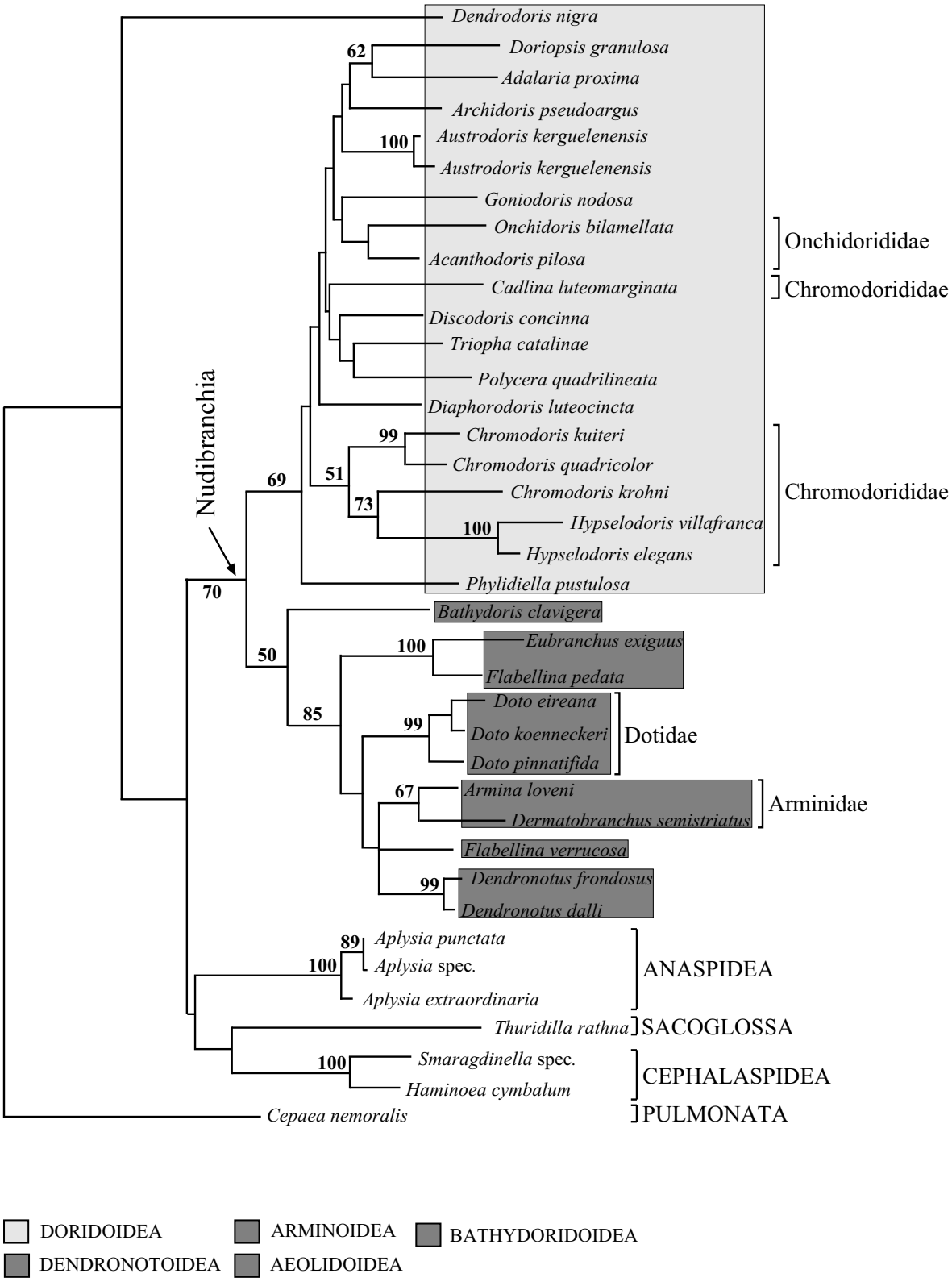


Fig. 4. Partial 16S rDNA, Neighbour-Joining tree (Kimura 2-parameter model, with pairwise deletion of gaps, transitions and transversions included), with bootstrap values of the parsimony analysis (only where higher than 50). Shading signifies the four major taxa described by Odhner (1934). Family-level and higher taxa groupings indicated with brackets to the right of the species names.

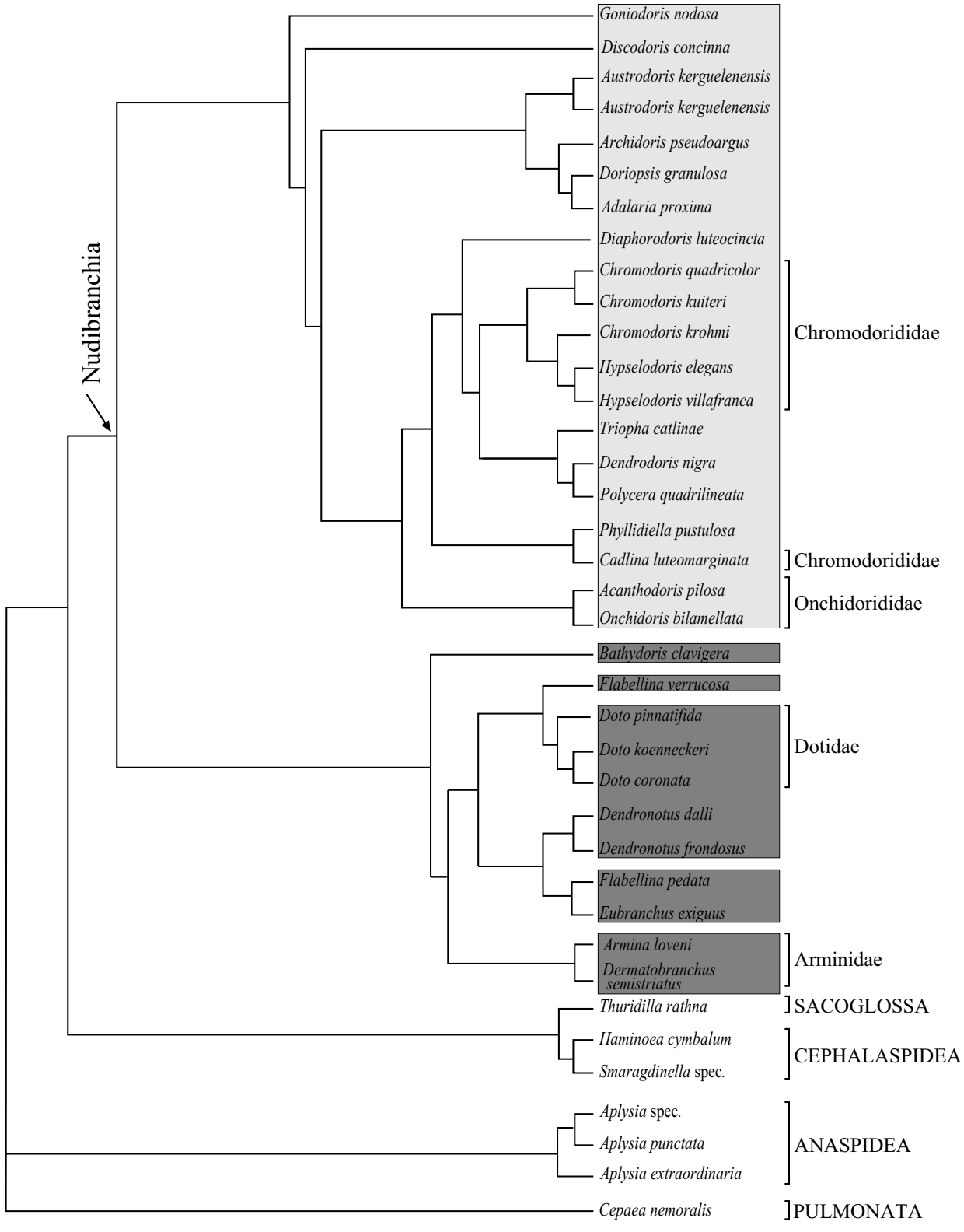
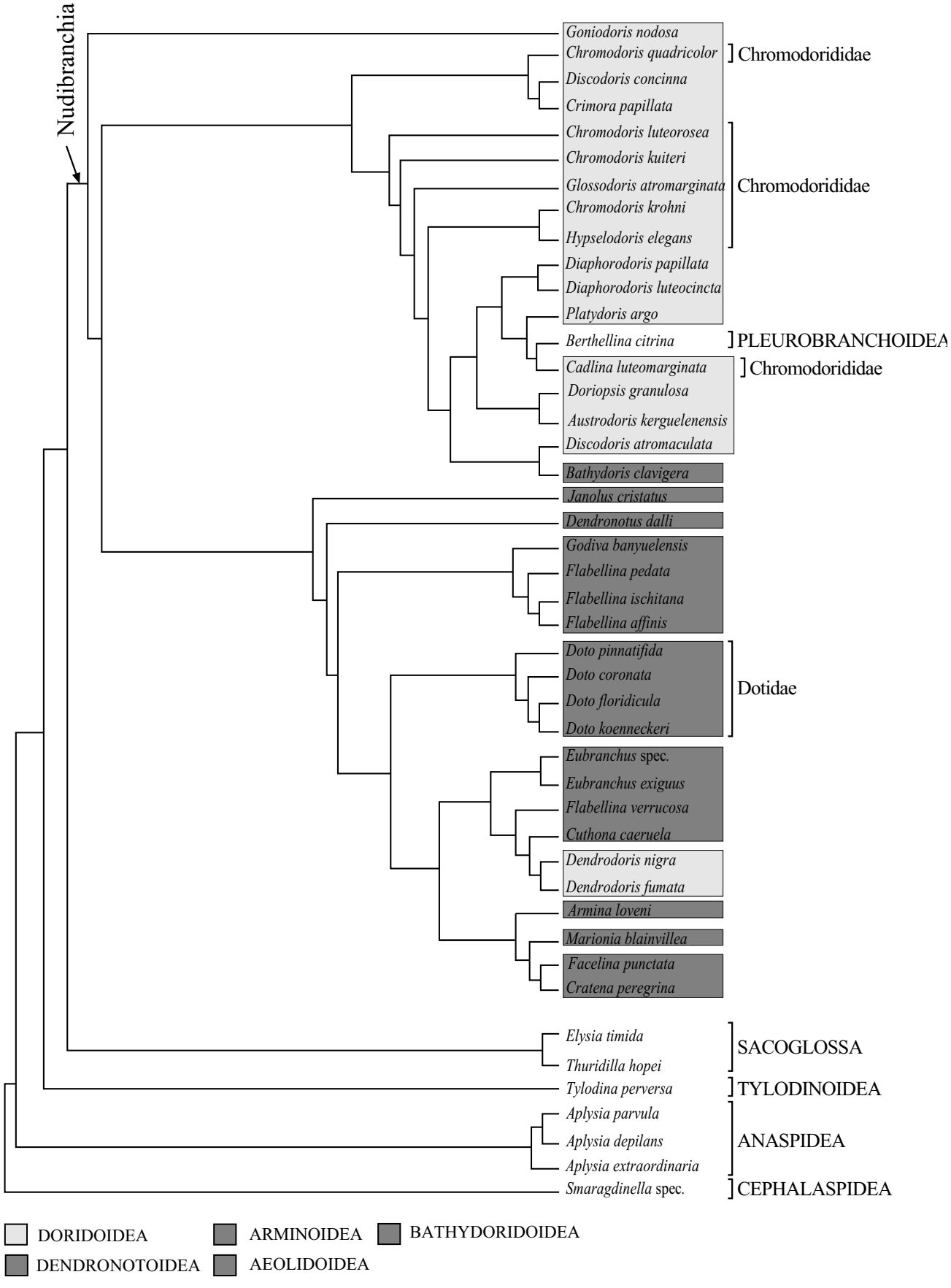


Fig. 5. Partial 16S rDNA, Maximum Likelihood tree, transition/transversion ratio of 1; input of sequences ordered, Ln Likelihood = -7584.39485. Shading signifies the four major taxa described by Odhner (1934). Family-level and higher taxa groupings indicated with brackets to the right of the species names.


Nudibranchia
Goniodoris nodosa
Chromodoris quadricolor
Discodoris concinna
Crimora papillata
Chromodoris luteorosea
Chromodoris kuiteri
Glossodoris atromarginata
Chromodoris krohni
Hypselodoris elegans
Diaphorodoris papillata
Diaphorodoris luteocincta
Platydoris argo
Berthellina citrina
Cadlina luteomarginata
Doriopsis granulosa
Austrodoris kerguelenensis
Discodoris atromaculata
Bathydoris clavigera
Janolus cristatus
Dendronotus dalli
Godiva banyuelensis
Flabellina pedata
Flabellina ischitana
Flabellina affinis
Doto pinnatifida
Doto coronata
Doto floridicula
Doto koenneckeri
Eubranchus spec.
Eubranchus exiguus
Flabellina verrucosa
Cuthona caeruela
Dendrodoris nigra
Dendrodoris fumata
Armina loveni
Marionia blainvillea
Facelina punctata
Cratena peregrina
Elysia timida
Thuridilla hopei
Tylodina perversa
Aplysia parvula
Aplysia depilans
Aplysia extraordinaria
Smaragdinella spec.
Chromodorididae
Chromodorididae
PLEUROBRANCHOIDEA
Chromodorididae
Dotidae
SACOGLOSSA
TYLODINOIDEA
ANASPIDEA
CEPHALASPIDEA
DORIDOIDEA
ARMINOIDEA
BATHYDORIDOIDEA
DENDRONOTOIDEA
AEOLIDOIDEA

tions 920 and 1145. Whether this insertion is a character typical for all Pleurobranchoidea has to be clarified by analysing more pleurobranchoid sequences. No 16S sequences of pleurobranchoids could be recovered, as degradation of specimen DNA made amplification impossible.

In many analyses using the 18S and 16S genes, the species from all other opisthobranch groups (Sacoglossa, Cephalaspidea and Anaspidea) are united as a sister group to the Nudibranchia/Nudipleura clade. Results on these groupings are preliminary due to lack of information on many other opisthobranch taxa.

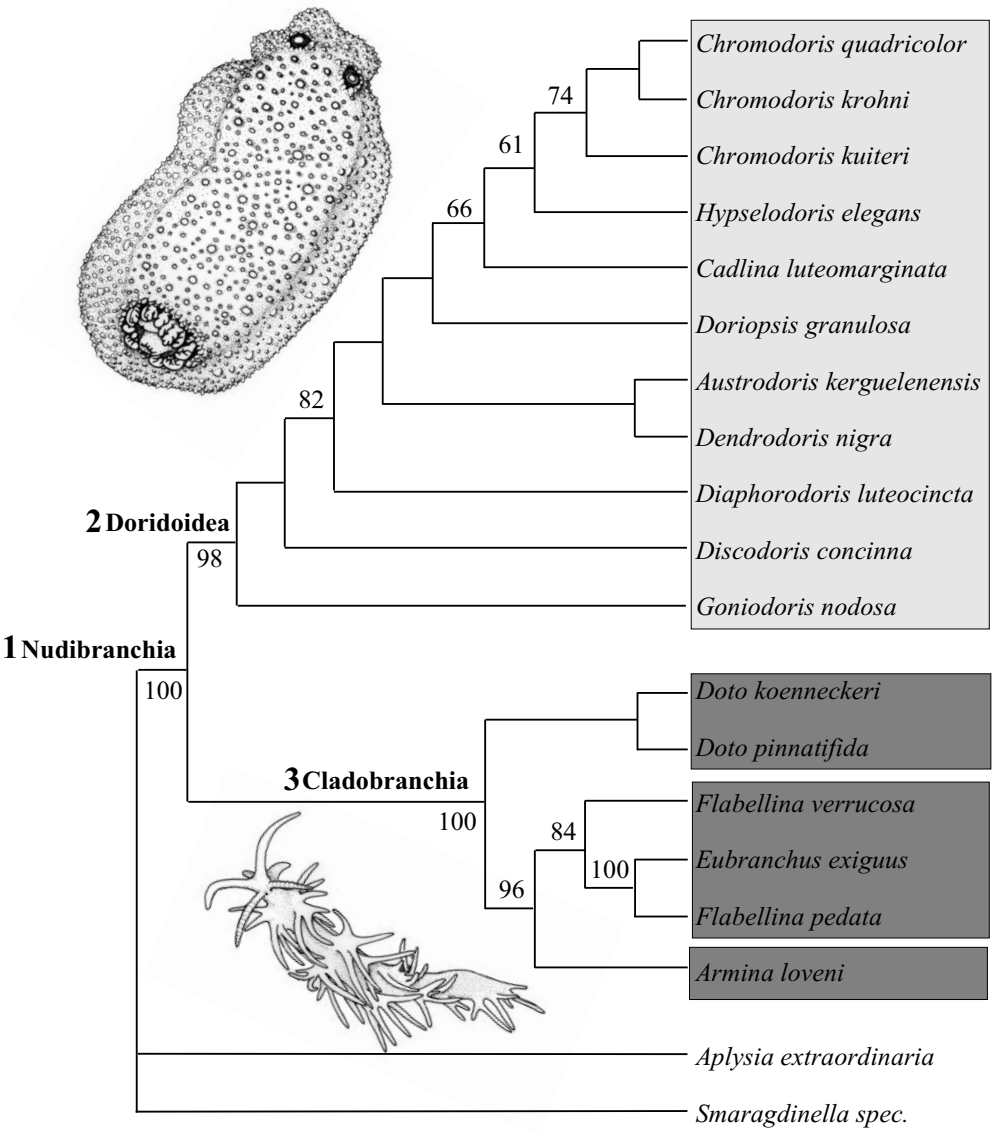


Fig. 7. Combined genes, Maximum Parsimony consensus tree (50% majority-rule, 3 shortest trees, tree length: 1058 steps) (heuristic search, tree bisection reconnection, CI: 0.81, HI: 0.2, RI: 0.85). Inserts show representatives of the major lineages (top: *Austrodoris kerguelenensis*, bottom: *Flabellina pedata*). Numbers indicate clades with following apomorphies discussed by Wägele & Willan (2000): 1 **Nudibranchia**: rhinophores solid, shell absent (through loss), pericardial complex oriented longitudinally, specialized vacuolated epithelium present. 2 **Doridoidea**: oesophagus without any cuticular lining, reproductive system triaulic, blood gland situated next to genital system or on top of cerebro-pleural complex, gill glands present. 3 **Cladobranchia**: primary gills (ctenidium) absent (through loss), jaws aliform, bursa copulatrix absent (through loss), blood gland absent (through loss). Shading signifies the four major taxa described by Odhner (1934) (for explanation see Figs 2–6).

◄

Fig. 6. Partial *cox1* gene, Maximum Likelihood tree using PROTPARS (PHYLIP) based on amino acid sequences. Shading signifies the four major taxa described by Odhner (1934). Family-level and higher taxa groupings indicated with brackets to the right of the species names.

In nearly all rDNA trees, two major clades appear within the Nudibranchia: the Doridoidea lineage and the Cladobranchia lineage. Within the clade Doridoidea, short branch lengths are observed in 18S rDNA NJ (Fig. 2) and ML phylograms (trees not shown). Additionally, the MP analyses resulted in several unresolved polytomies and low bootstrap values (Fig. 3; bootstrap values lower than 50 not shown). The evolutionary rate of the 18S rDNA in Doridoidea is significantly higher than the evolutionary rate observed in the other major lineage, the Cladobranchia (18S rDNA: Z-value 12.52, CP = 99.96%). This result is confirmed by considering the *cox1* sequences of the Anthobranchia and the Cladobranchia lineages in a relative rate test. These sequences also appear to have evolved at significantly different rates (*cox1*: Z-value 3.27, CP= 99.88%). In the 18S rDNA NJ (Fig. 2) and ML (not shown) phylograms, long branches separate especially Dendronotoidea taxa within the Cladobranchia. For *Tritonia nilsodhneri* and the genus *Doto,* significantly higher evolutionary rates could be observed (Z-values: 5.81, CP: 99.96%, and 4.37, CP: 99.96%, respectively).

Some species show an affiliation to both of the two major nudibranch clades, when different methods and markers are compared. In the 16S analyses *Bathydoris clavigera* (not included in the 18S analysis due to degradation of DNA quality) usually appears as sister taxon to the Cladobranchia (NJ, BT, ML) (Figs 4–5). However, when considering transitions only in a NJ analysis (not figured), *B. clavigera* is the sister taxon to the Doridoidea, whereas in the MP analysis it is the sister taxon to all other Nudibranchia. *Jorunna tomentosa*, a cryptobranch doridoidean, is found alternatively to be sister taxon either to the Cladobranchia (Figs 2–3) or to the Doridoidea (NJ, transitions only; not figured). The 18S rDNA sequence of this species diverges extremely (by 12%) from all other Doridoidea sequences (next-highest sequence divergence 4%). Investigation of its sequence in the alignment by eye revealed several inversions of nucleotide sequences comprising two to three base pairs. The position of the genus *Dendrodoris* varies considerably among different trees from the three different markers. Whereas it is assigned to the Doridoidea as a rather basal taxon in all 18S analyses (Figs 2–3) and the ML analysis of the 16S (Fig. 4), it is positioned as the sister taxon to all Opisthobranchia in the MP and NJ analyses of the 16S (Fig. 4), and grouped within the Cladobranchia when investigating the *cox1* gene. The *Dendrodoris* 16S and *cox1* sequences diverge from other Doridoidea sequences by about 30 to 40%, respectively. Similar results are obtained for *Goniodoris nodosa*. This phanerobranch species is placed as sister taxon to all doridoidean species (18S all analyses, 16S ML, combined analysis; Figs 2–3, 5, 7), or as sister taxon to the family Onchidorididae (16S NJ; Fig.4), or as sister taxon to all nudibranchs (*cox1*, Fig. 6).

Relationships within each of the two major nudibranch clades differ depending on the data sets and phylogenetic methods used. No congruent solutions could be found within the Doridoidea, with the exception of certain genus-level relationships. For instance, comparisons of 18S and 16S sequences (Figs 2–5) indicate monophyly, whereas those of *cox1* (Fig. 6) suggest paraphyly for the morphologically well-defined family Chromodorididae (*Cadlina* excluded). The Onchidorididae are monophyletic according to the 18S and 16S data. Comparing results from the three markers within the Cladobranchia, there is also a high level of incongruence between the different analyses. According to the 18S data, the Aeolidoidea are monophyletic (Figs 2–3), whereas the Dendronotoidea (with *Tritonia*, *Tritoniella*, *Melibe*, *Dendronotus*, and Dotidae) and the Arminoidea (with *Janolus* and Arminidae) are paraphyletic. According to the 16S rDNA, neither the Aeolidoidea nor the Dendronotoidea are monophyletic (Figs 4–5). The *cox1* sequences suggest paraphyly or even polyphyly for all three cladobranch taxa (Fig. 6). On the family and genus levels, the 18S gene resolves only the Dotidae and Arminidae, whereas the 16S and *cox1* genes indicate the monophyly of morphologically well defined families and genera, e.g. Dotidae, Arminidae, *Dendronotus*, *Eubranchus*. Only the families Arminidae and Dotidae are supported by all our analyses. The genus *Flabellina*, represented with two species in the 18 analysis, is rendered paraphyletic.

In the combined analysis of the three markers (Fig. 7), monophyly is supported for each of the clades Nudibranchia, Doridoidea, Cladobranchia, and Aeolidoidea. Not enough genera are included to evaluate the Dendronotoidea and „Arminoidea“.

Discussion

The 18S rDNA, 16S rDNA, and *cox1* comparisons in this work represent the largest, most comprehensive molecular data set available for the Nudibranchia. The identical topology concerning the major lineages, that resulted from MP, NJ, and ML phylogenetic analyses of the 18S rDNA, 16S rDNA, and *cox1* gene data sets and from the combined analysis of these three markers, is also supported by high bootstrap values. Thus, the presence of clear and congruent phylogenetic signals from several molecular loci supports the hypothesis of a common ancestor for all Nudibranchia. This confirms the results of previous cladistic analyses based on morphological and histological (Wägele 1997, Wägele & Willan 2000) as well as molecular data (Wollscheid & Wägele 1999).

Schmekel (1985) proposed the opisthobranch taxon Pleurobranchoidea as the sister taxon of the Nudibranchia. This was supported by Wägele (1997) and

Wägele & Willan (2000) who identified two synapomorphic features: the possession of a blood gland, and the loss of the osphradium (a sensory organ in the mantle cavity). Wägele & Willan (2000) introduced the new name Nudipleura for this Nudibranchia/Pleurobranchoidea clade. The sister taxon relationship is confirmed by the data from the 18S rDNA analysis, with two members of the Pleurobranchoidea included. Nevertheless, information on more opisthobranchiate taxa is needed to test the hypothesis of monophyly of the Nudipleura and to elucidate the relationships of this taxon within the Opisthobranchia.

Thollesson (1999a) – analysing ten nudibranch, one pleurobranchoid (*Berthella*), and seventeen other gastropod species in his 16S rDNA analysis of the Euthyneura - found a sister taxa relationship of *Berthella* with the Cladobranchia. Our data on *cox1* places the closely related pleurobranchoid genus *Berthellina* as a member of the Anthobranchia. 16S rDNA and *cox1* are molecular markers usually applied at higher taxonomic levels in phylogenetics. Statistical analysis of the 16S rDNA and *cox1* showed a saturation of substitutions, mainly transitions, for distantly related species. Thus, the position of *Berthellina citrina* within the Anthobranchia is most likely due to homoplasy, especially as there are no morphological features supporting a *B. citrina*/Anthobranchia relationship.

The Nudibranchia divide into two major monophyletic clades, the Anthobranchia (= Doridoidea + Bathydoridoidea) and the Cladobranchia. The 18S rDNA, 16S rDNA and *cox1* genes, as well as the combined analysis with a reduced number of species, provide consistently good support for this sister taxon relationship which is maintained even when adding or removing species from the data set or using different optimality criteria in the analyses. This conforms with the conclusions of Thollesson (1999a) and Wollscheid & Wägele (1999) based on molecular data, and also with the findings of Wägele & Willan (2000). Members of the Cladobranchia show the loss of primary ctenidial gills (thus the alternative group name, Actenidiacea) and reduction of other features, and they prey mainly on cnidarians. Representatives of the Anthobranchia possess primary gills (thus: Ctenidiacea) and tend to feed on incrusting invertebrates such as sponges or bryozoans. The varying and incongruent positions of some species in the present analyses, which are morphologically well characterized and have quite obvious relationships among the Nudibranchia and Opisthobranchia (*Berthellina citrina, Jorunna tomentosa, Dendrodoris nigra* and *D. fumata*), should not lead to questioning the monophyly of the major clades, but will have to be considered more thoroughly when more related taxa (e.g., other species of the genera in question, or of the same family) are included in molecular phylogenetic analyses.

Wägele (1989) separated the cold-water nudibranch genus *Bathydoris* from the Doridoidea and gave it separate status equal to the latter. This was supported by Wägele & Willan (2000). In our analyses, *Bathydoris clavigera* appears as sister taxon of either the Doridoidea or the Cladobranchia or the Nudibranchia in general. This partly contradicts interpretations of morphology, in which Bathydoridoidea and Doridoidea share several derived features. Members of both groups possess an elongate anterior notum which encloses the rhinophores due to anterior extension and overgrowth of the head. In addition, the anus, the nephroproct and the gills have migrated to a mediodorsal position. The placement of *Bathydoris clavigera* in some of our molecular analyses as sister taxon to the Cladobranchia (Fig. 5) could be due to its extremely divergent sequence (16S rDNA: 18–32% divergence from the other Doridoidea). In addition, the relative rate test revealed a higher evolutionary rate of the *cox1* gene of *B. clavigera* compared to all other doridoidean species. The high incongruence of the results shows that the phylogenetic signal in the genes 16S and *cox1* is not strong enough to resolve the placement of the Bathydoridoidea within the opisthobranchiate system. An analysis of other molecular loci, especially 18S rDNA, would be of high value to determine whether the position of this species depends on the sequence under investigation or needs to be reinvestigated by other morphological features.

Within the Doridoidea, a phylogeny at the family or genus level, that is congruent with our knowledge on morphology, can be obtained best when analysing the 18S and 16S rDNA. But not all such taxa that have been defined morphologically are recognizable in our molecular topologies. Most Chromodorididae form a clade, but the genus *Cadlina* does not group within this family. Similar results were obtained by Thollesson (1999b) from the 16S rDNA of 24 doridoidean species. In the combined analysis, *Cadlina* is the sister taxon to all other chromodorids, but this might be due to the reduced number of doridoideans and does not necessarily imply its basal position within the Chromodorididae. According to Rudman (1984), the presence of mantle dermal formations is a synapomorphy that unites *Cadlina* with the Chromodorididae. Mantle dermal formations are now known from several other nudibranch taxa, e.g. *Limacia clavigera*, and even from sacoglossans (*Placobranchus ocellatus*, unpublished results of senior author). Therefore, a thorough analysis of the Chromodorididae and related taxa based on morphological and histological features is needed, and a reevaluation of the position of *Cadlina* necessary. The family Onchidorididae as well as the genera *Dendrodoris*, *Hypselodoris*, *Chromodoris* and *Discodoris* each usually reappear as clades, independent of the reconstruction method, although bootstrap values are sometimes lower than 50

(not all results figured). The time between speciation events for these groups may have been too short to establish a stronger phylogenetic signal by the applied molecular markers. Noteworthy is the absence of any signal for the clades Cryptobranchia and Phanerobranchia, as well as the family Doriididae which traditionally comprises (amongst others) the investigated genera *Austrodoris*, *Archidoris*, *Discodoris* and *Platydoris*. Concerning the 16S data, Thollesson (1999b) described similar results. Conclusions on phylogeny and evaluation of these taxa are preliminary, since results from the three molecular markers are too divergent and no cladistic analysis based on morphology is available.

Jorunna tomentosa is a member of the Doridoidea based on a number of synapomorphies (i.e., triaulic genital system, blood gland next to or on top of cerebropleural complex, oesophagus without any cuticular lining, gill glands present; Wägele & Willan 2000). However, it also shows some special internal features, such as the mantle rim organs with unknown function, and a modified radula (Foale & Willan 1987, Wägele 1998). *J. tomentosa* possesses derived molecular features (inversions) in the 18S sequence, which distinguishes this species from all other Doridoidea and which resulted in wide separation from the other Doridoidea. Further studies on this genus will show whether these findings are artefacts or typical for the species, or even the genus.

The position of *Dendrodoris* varies to a high degree depending on the method and marker used. *Dendrodoris* shows the synapomorphies of the Doridoidea already mentioned above (Wägele et al. 1999). On the other hand, *Dendrodoris* has many unique characters distinguishing it from other doridoids, such as the lack of the specialized vacuolated epithelium, the lack of jaws and radula, huge oral glands, and small salivary glands (Wägele et al. 1999). Its 18S rDNA unequivocally places *Dendrodoris* within the Doridoidea, confirming the morphological hypothesis, whereas phylogenetic reconstructions with 16S rDNA and *cox1* contradict this hypothesis. No significantly higher substitution rate in these genes could be recognized for *Dendrodoris nigra*. The 16S rDNA as well as *cox1* of *Dendrodoris* diverge to a high degree from the comparable sequences of all other doridoidean taxa. *Dendrodoris* may have branched off early in the doridoidean radiation, then accumulated many mutations at least in the 16S rDNA and *cox1* genes, and thus lost the signal to group it with the Doridoidea. To resolve this, more *Dendrodoris* species will have to be examined, probably using new molecular markers.

When analysing the 18S rDNA data set and the combined gene set, only the taxon Aeolidoidea is confirmed as monophyletic within the Cladobranchia. When considering 16S rDNA and *cox1* sequences, the branching of the Aeolidoidea depends on the number of species and choice of taxa, a fact which may strongly influence the results (Lecointre et al. 1993). For the 16S rDNA data set, the number of species for the Aeolidoidea, and Cladobranchia in general, seems to be too low to infer phylogenetic relationships with confidence. When analysing *cox1* sequences, the paraphyly of the Aeolidoidea is a result of the small amount of analysed species, especially when considering the high variability of these sequences. Wägele & Willan (2000) considered the Aeolidoidea to be monophyletic, with the synapomorphic presence of cnidosacks in dorsal appendages where the cnidocysts of the prey are stored and utilised for defence. The genus *Flabellina* comprises more than 100 species with a wide range of plesiomorphic and apomorphic characters in the different species. According to Gosliner & Kuzirian (1990), *F. verrucosa* represents a basal, and *F. pedata* a derived species. The results of the 18S and the combined analysis indicate paraphyly for the genus. Inclusion of many more members of aeolidoidean families as well as *Flabellina* species is needed to clarify the putative paraphyly of the genus and the relationships of the highly variable species amongst Aeolidoidea.

The paraphyly of the Dendronotoidea is partly consistent with conclusions based on morphological features (Wägele & Willan 2000). Our molecular-based results confirm both the monophyly of Dotidae and its exclusion from the Dendronotoidea, but not even the remaining dendronotoidean species offer support for a monophyletic taxon in the sense of Wägele (1997) and Wägele & Willan (2000). These authors discussed the following synapomorphies for uniting all dendronotoidean taxa except the Dotidae: presence of tentacular extensions on the oral veil, presence of rhinophoral sheaths, possession of a cuticle lining the stomach. Reconstructing the phylogeny with the 18S rDNA sequences, the Dendronotoidea appear not only paraphyletic, but the taxa are also separated from all other Cladobranchia through long branches (Fig. 2). Long branches appear when taxa evolve at a higher rate than their sister taxa, as is probably the case for *Tritonia* species and the genus *Doto*, thus showing higher divergence from the ground pattern of the last common ancestor (Swofford et al. 1996, Hendy & Penny 1989).

The molecular data confirm the paraphyly of the Arminoidea that also had been concluded from morphological data (Wägele & Willan 2000). However, the two sets of data differ in suggested ancestry for the „Arminoidea“ species. Kolb & Wägele (1998) have performed a thorough phylogenetic analysis of the family Arminidae based on morphological and histological characters. That family's characteristic autapomorphy is the presence of marginal sacs in the lateral notum. Our 18S and 16S analyses confirm the monophyly of the Arminidae. Only one *cox1* sequence for the Arminidae was available, therefore no results can be obtained from this gene here.

The 18S rDNA gene is generally considered to resolve older speciation events and coincides best with the phylogeny of Nudibranchia proposed by Wägele & Willan (2000) based on morphological and histological data. The 18S rDNA results are highly robust when using different phylogenetic methods. On the family and genus levels the resolution of relationships is lower in the Cladobranchia than in the Doridoidea.

Partial 16S rDNA and *cox1* genes have been used to address phylogenetic questions on family, genus or even population levels (e.g., Simon et al. 1994, Thollesson 1999a, Reid et al. 1996, Lydeard et al. 1997, Remigio & Blair 1997). In our analysis, these parts of the genes did not provide considerably better resolution at the family or genus level than the 18S rDNA.

The data presented here contribute to our understanding of the relationships of nudibranch taxa. They confirm the monophyly of the Nudibranchia. Within the latter, they generally support the assumption of two major lineages (Fig. 7) which are also very different morphologically. However, only the Anthobranchia clade is characterized by apomorphic features which are not mere reductions (notum overgrowing head and enclosing rhinophores during ontogeny; postero-median site of anus, nephroproct and gills; presence of a caecum – see Wägele & Willan 2000). In contrast, the Cladobranchia still show many plesiomorphic features, and the group's monophyly is manifested mainly in reduction of characters (loss of primary gills; loss of bursa copulatrix; loss of blood gland - see Wägele 1997, Wägele & Willan 2000). Here the molecular data are useful in evaluating the conclusions based on morphological data, especially in those cases with congruencies in all three genes. However, many of the lower-level relationships are not well resolved by our choice of molecular markers, and some taxa that are well supported by comparative morphology and other biological data (e.g., the Aeolidoidea) are weakly or not supported by the molecular phylogenies based on the three markers. We conclude that a critical evaluation of the three different markers in the light of morphological data and hypotheses is necessary, nevertheless all data sets serve to enrich the understanding of phylogeny and evolution of the Nudibranchia. Incongruence between different data sets encourages further research for reliable results through analysing more taxa, other molecular markers, and new morphological and histological data.

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