Title

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

Journal

Organisms, Diversity and Evolution, 1(4)

Author

Wagele, Heike

Publication Date

2001-12-31

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

Evi Wollscheid-Lengeling[1], Jeffrey Boore[2,3], Wesley Brown[2], Heike Wägele[1]

[1]Ruhr-Universität Bochum, Lehrstuhl f. Spezielle Zoologie, 44780 Bochum, GERMANY

[2]The University of Michigan, Department of Biology, 830 N. University, Ann Arbor, Michigan 48109-1048, USA

[3] DOE Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, California 94598 USA

Corresponding author:

Dr. Heike Wägele

Spezielle Zoologie

Ruhr-Universität Bochum

44780 Bochum

Germany

Phone: + 49-234-3224585

fax: + 49-234-14114

email: Heike.Waegele@ruhr-uni-bochum.de

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 are 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 designated as butterflies of the ocean because of their attractive colours and body forms. They live in exclusively 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 manifolded 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* (chemical defenses 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 on these gastropods showed this group to be paraphyletic and demonstrated the close relationship of some prosobranchs, Valvatoidea and Architectonicoidea, with the Euthyneura, comprising the group Heterobranchia. Further, both the monophyly of the Pulmonata and that of the Opisthobranchia remain uncertain (e.g. Haszprunar, 1988; Tillier et al., 1994, Mikkelsen, 1996; Ponder & Lindberg, 1997, Winnepennickx, et al. 1998) (Fig. 1).

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 (Fig. 1). This was corroborated by Wollscheid & Wägele (1999) by a comparison of the complete 18S rDNA sequences of 53 gastropods, including 19 nudibranch taxa. However Thollesson (1999a) concluded that they are paraphyletic based on his comparison of part of the 16S rDNA (approximately 480 bp) of nearly 30 gastropods.

Within the Nudibranchia (Fig. 1), two major groups (Cladobranchia and Anthobranchia) have been recognized for nearly 200 years (Férussac, 1822). At a lower taxonomic level, Odhner (1934) advanced three major taxa within the Cladobranchia, the Dendronotoidea, Arminoidea and Aeolidoidea. Within the Anthobranchia, he recognized only the subordinate taxon Doridoidea. Wägele (1989) discussed an additional order the Bathydorididae (former members of the Doridoidea) as the sistertaxon of the Doridoidea.

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

These controversial 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.

Comparisons of these nucleotides and inferred amino acid sequences are used to address the monophyly of Nudibranchia and the derivation of its subordinate taxa. This is the largest dataset to date for addressing these phylogenetic questions.

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). These studied taxa, along with their locations of collection and the database accession numbers for their sequences are shown in table 1. The alignments are available at the homepage of Heike Wägele (www.ruhr-uni-bochum.de/spezzoo/heike).

The 18S rDNA fragments were amplified using primers matching conserved regions (18A1: 5'CCT ACT CTG GTT GAT CCT GCC AGT) and (1800: 5'TAA TGA TCC TTC CGC AGG TT) using PCR (38 cycles of 30s at 94° C, 50s 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 protocoll the later 18S rDNA fragments were sequenced directly. For the 18S rDNA only one clone/DNA fragment was sequenced for each specie. 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 analyzed, 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). 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, both options: Complete Deletion of gaps as well as Pairwise Deletion of gaps were 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 neighbor interchange or alternatively tree bisection reconnection; 50% majority-rule consensus tree. Bootstrap

analyses contained 1000 replicates, gaps treated as missing ML analyses of the sequences were performed exclusively with DNAML (Phylip) with following settings for DNA sequences: search for best tree, use empirical base frequencies, four categories of substitution rates (0,5; 1; 2 and 5; these substitution rates have been 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. The results of these analyses did not differ in great detail from the analyses with input of sequences by order. A parsimony analysis for protein sequence data has been performed by applying PROTPARS (PHYLIP) with settings for inferred amino acid sequences: use threshhold parsimony: no, analyze 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 case of the 18S rDNA representatives (*Littorina*) of the sistergroup 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 Caenogastropods were not available for the *cox1* analyses, thus a species (*Smaragdinella)* investigated here and belonging to the basal Cephalaspidea s. str. (Mikkelsen 1996) was used to root the tree. Finally, to avoid misinterpretations by using different species as outgroups, phylogenetic analyses of the Nudibranchia were also performed by including only opisthobranch taxa and using *Smaragdinella* as the outgroup for rooting. Only those 19 species have been included in the combined analysis of the three markers, where information on all markers was available. The DNA sequences of the *Cox I* gene were used in the combined analysis.

Evolutionary rate variation was assessed using LINTRE (Takezaki et al., 1995) following the Wu & Li (1985) relative rate test. The nucleotide substitution rates among the species were compared with respect to the mentioned outgroups.

Results

The alignment resulted in 2,468 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 favoring A+T. The differences in base composition biases between species under consideration were not significant (χ^2 test: p= 0.000000 for 18S rDNA, χ^2 test: p=0.000137, χ^2 test: p=0.000115), thus compositional bias should not have interferred with the recovery of phylogenetic signal. The alignment of 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. Thus, 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 of higher taxonomic level. Various TS/TV ratios (0.5, 1, 2 and 5) were used in the ML analyses which yielded identical or congruent topologies; thus, only one ML tree for the 16S rDNA (Fig. 5) and *cox1* genes (Fig. 6) is shown respectively.

The robustness of these results is supported by the high bootstrap values obtained in the MP analyses (Fig. 3-5 and additional trees not shown). Choosing *Smaragdinella* as outgroup in the 18S and 16S analyses did not effect the topology of the trees and was therefore not considered further.

The analyses of the 18S and 16S data sets, using all different phylogenetic methods with the different options and settings as mentioned in the Material and Methods, support a monophyletic Nudibranchia clade (Fig. 2-5). In the 18S analyses the two members of the Pleurobranchoidea are sister taxa to the Nudibranchia. Both species investigated here are characterized by a long insertion between positions 920 and 1145. Whether this insertion is a character typical for all Pleurobranchoidea has to be clarified by analyzing more pleurobranchoid sequences. In the *cox1* analyses (DNA sequences, as well as amino acid sequences) the position of the pleurobranchid species *Berthellina citrina* varies and renders the Nudibranchia paraphyletic (Fig. 6). The 16S sequence of pleurobranchids was not investigated, due to degradation of DNA quality, which made amplification impossible.

In all trees, two major clades appear within the Nudibranchia: the Doridoidea lineage and the Cladobranchia lineage. The position of *Bathydoris clavigera* (not included in the 18S analysis) varies according to the phylogenetic methods used. In the 16S analyses this species usually appears as sister taxon to the Cladobranchia (NJ, BT, ML) (Fig. 4, 5). When considering transitions only in a NJ analysis, *B. clavigera* is the sister taxon to the Doridoidea (data not shown), whereas in the MP analysis it is the sister taxon to all other Nudibranchia.

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). The evolutionary rate of the 18S rDNA of the Doridoidea species 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 considering the *cox1* sequences of the Anthobranchia and the Cladobranchia lineages in a relative rate test. In this case as well the *cox1* sequences evolve at significantly different rates (*cox1*: Z-Value: 3.27, CP= 99.88%).

In the 18S rDNA NJ (Fi.g 2) and ML phylograms (tress not shown) long branches separat especially Dendrontotidea taxa within the Cladobranchia. For *Tritonia nilsodhneri* and genus *Doto* significantly higher evolutionary rates (Z-Value: 5.81, CP: 99.96% and Z-Value: 4.37, CP: 99.96% respectivly) could be observed.

The relationships within 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 indicate monophyly (Fig. 2-5) whereas those of *cox1* suggest paraphyly (Fig. 6) of the morphologically well-defined family Chromodorididae (*Cadlina* excluded). Additionally *Jorunna tomentosa*, traditionally considered a typical dorid representative, is found sometimes to be sister taxon to the Cladobranchia (Fig. 2-3) as well as sister taxon to the Doridoidea (NJ, transitions only, data not shown). The 18S rDNA sequence of this species diverges extremely compared to all other Doridoidea sequences (12%, in contrast to the highest sequence divergence within the Doridoidea without *J. tomentosa*, which is 4%).

In the 16S analyses, using distance and parsimony methods, the dorid genus *Dendrodoris* appears as the sister taxon to all opisthobranchs (Fig. 4). Only the ML analysis supports the affiliation with the Doridoidea (Fig. 5). In the *cox1* analyses *Dendrodoris* is located within the Cladobranchia. The *Dendrodoris* 16S and *cox1* sequences diverge from other Doridoidea sequences by about 30 and 40%, respectively.

Comparing the results of the three markers within the Cladobranchia, there is even greater conflict between ich weiss hier nicht was Du schreiben wolltest. Analyzing the 18S data, the Aeolidoidea are monophyletic (Fig. 2-3), whereas the Dendronotoidea (with *Tritonia, Tritoniella, Melibe*, *Dendronotus* and Dotidae) are paraphyletic, as well as the Arminoidea (with *Janolus* and Arminidae). Independent of the method, the 16S rDNA or *cox1* sequences suggest paraphyly or even polyphyly for all three cladobranch taxa (Figs. 4-7). Taxa which have been grouped within a family or genus by morphological features do not branch together analysing the 16S rDNA or *cox 1*, because of a lack of similar features within the sequences. Only the families Arminidae and Dotidae are supported by our analyses.

In the combined analysis of the three markers, the monophyly of the clades Nudibranchia, Doridoidea, Cladobranchia and Aeolidoidea is supported (Fig. 7).

Discussion

The 18S rDNA, 16S rDNA and *cox1* comparisons presented in this work are the largest molecular data set available for 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 the combined analysis of these three markers is also supported by high bootstrap values. Thus the presence of a clear phylogenetic signal from several molecular loci congruently support the hypothesis of a common ancestor for all Nudibranchia. This confirms the results of previous cladistic analyses on the phylogeny of the Nudibranchia based on morphological and histological data (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 (1987) and Wägele & Willan (2000) who identified two synapomorphic features, the possesion 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 sistertaxon relationship is confirmed by the data of the 18S rDNA analysis, with two members of the Pleurobranchoidea included. Nevertheless, inclusion of many more species of the other opisthobranchiate taxa is needed, to strengthen the hypothesis

of monophyly of the Nudipleura and especially the relationship of this taxon within the Opisthobranchia.

Thollesson (1999a), analyzing 10 nudibranch, one pleurobranchid (*Berthella*) and 17 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 this specie as a member of the Anthobranchia. 16S rDNA is a molecular marker usually applied for higher level in phylogenetics, as well as *cox1*. Statistical analysis of the 16S rDNA and *cox 1* showed a saturation of substitutions, mainly transitions for distantly related species. Thus the position of *Berthellina citrina* within the Anthobranchia can be concluded due to homoplasy and not as a sequence similarity due to a common ancestor. Especially, as there are no morphological features supporting a *B. citrina* / Anthobranchia relationship.

The Nudibranchia branch into two major monophyletic clades, the Doridoidea or Anthobranchia (=Doridoidea and Bathydoridoidea) and Cladobranchia. The branching pattern is maintained even when adding or removing species from the data set or by 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). The Cladobranchia, with loss of primary ctenidial gills (therefore sometimes called Actenidiacea) and reduction of other features, prey mainly on cnidarians. The Anthobranchia, retaining the primary gills (therefore often called Ctenidiacea), tend to feed on incrusting invertebrates, such as sponges or bryozoans. The 18S rDNA, 16S rDNA and *cox 1* genes provide consistently good signal for an Anthobranchia / Cladobranchia sistertaxon relationship.

In former times, the monogeneric Bathydorididae have been assigned to the Doridoidea, until Wägele (1989) showed that this cold water nudibranch taxon has a separate evolutionary line from the Doridoidea and so gave it equal status to the Doridoidea within the Anthobranchia. This was also supported recently by Wägele & Willan (2000) in their cladistic analysis. In our analyses presented here, *Bathydoris clavigera* appears as sister taxon of either the Doridoidea (16S: NJ, transitions only; *cox1*: MP, ML) or the Cladobranchia (16S: NJ, ML, bootstrap analysis; *cox1*: NJ,), or the Nudibranchia in general (16S: MP). This is partly in contradiction with morphological results, where Bathydoridoidea and Doridoidea share several derived features. Both possess an elongated anterior notum, which encloses the rhinophores due to anterior extention and overgrowth of the head. Furthermore, in both taxa the anus, the nephroproct and the gills have migrated to a mediodorsal position.

The position of *Bathydoris clavigera* in our molecular analyses as sistertaxon to the Cladobranchia (Fig.5) could be due to its extremely divergent sequence (16S rDNA: 18-32% sequence divergence compared to all other Doridoidea sequences). Additionaly the relative rate test revealed a higher evolutionary rate of the *cox1* gene of *B.clavigera* compared to all other doridean species. Thus either synapomorphic sequence positions have been substituted several times and the position as sistertaxon within the doridoidea is due to homoplasy (16S rDNA) or its positions as sistertaxon to the Cladobranchia is because of the evolution of new features in the *cox 1* gene (Fig. 6).

It will be of great interest to determine whether the position of this species depends on the sequence under investigation or needs to be reinvestigated by other morphological features. Therefore an analysis of other molecular loci, especially 18S rDNA, would be of value.

Within the Doridoidea, a phylogeny at the family or genus level can be obtained best when analyzing the 18S and 16S rDNA. But not all taxa that have been identified morphologically are recognized in our molecular topologies. The Chromodorididae form a clade, although the genus *Cadlina* does not group within this family (except in the combined analysis with a reduced number of doridoideans). Similar results are obtained by Thollesson (1999b) analyzing the 16S rDNA of 24 doridoidean species. According to Rudman (1984) the possession of mantle dermal formations is a synapomorphy that unites *Cadlina* with the Chromodorididae. But it has to be emphasized that a thorough analysis of the Chromodorididae and related taxa based on morphological and histological features is missing. The families Phyllidiidae and Onchidoridiidae, as well as the genera *Dendrodoris*, *Hypselodoris*, *Chromodoris* and *Discodoris* are usually recognized when using different methods for the two different data sets. Nevertheless the time between speciation events for these groups may have been too short to establish a stronger phylogenetic signal in these molecular markers. Noteworthy is the absence of any signal for the family Doridiidae, which traditionally comprise (amongst others) the investigated genera *Austrodoris*, *Archidoris*, *Discodoris* and *Platydoris* investigated here. Concerning the 16S data, Thollesson (1999b) came to the same conclusions.

Although *Jorunna tomentosa* is easily recognized as a member of the Doridoidea based on a number of synapomorphies (i.e., triaulic genital system, blood gland next or on top of cerebropleuralcomplex, oesophagus without any cuticular lining, gill glands present; Wägele & Willan, 2000), it also shows some special internal features, as there are the mantle rim organs with unknown function and a modified radula (Foale & Willan,

1987; Wägele, 1997/1998). *J. tomentosa* possesses derived molecular features in the 18S sequence which distinguishes this species from all other Doridoidea and which resulted in an exclusion from the Doridoidea in the phylogenetic analyses presented here.

The position of *Dendrodoris* varies to a great extent depending on the method and marker used. *Dendrodoris* is a typical dorid, with the apomorphies of the Doridoidea already mentioned above. Nevertheless, *Dendrodoris* also has many unique characters, which distinguishes this taxon from other dorids, such as the lack of the specialized vacuolated epithelium, the loss of jaws and radula, huge oral glands, and small salivary glands (Wägele et al., 1999). Its 18S rDNA possess features, which unequivocal group *Dendrodoris* within the Doridoidea, confirming the morphological hypothesis, whereas phylogenetic reconstructions with the 16S rDNA and cox 1 gene contradict this hypothesis. No significant higher substitutionrate of the 16S rDNA or *cox 1* gene of *Dendrodoris nigra* could be recognized. The s16S rDNA as well as *cox 1* of *Dendrodoris* diverge to a great extent compared to the 16S rDNA and *cox 1* sequences of all other doridean taxa. A hypotheses could be that *Dendrodoris* has been branched off at the beginning of the doridean radiation. Thus accumulating mutations in the 16S rDNA and *cox 1* gene with loosing the signal to group them within the Doridoidea. To proof this hypotheses more *Dendrodoris* species have to be examined, probably using new molecular markers.

When applying 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 seems to depend on

the number of species and taxa choice(Lecointre et al., 1993), a fact which can strongly influence the results. For the 16S rDNA data set the number of species for the Aeolidoidea and Cladobranchia, in general, seems to be too small to infer phylogenetic relationships with confidence. When analyzing *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 as monophyletic, supported by the presence of cnidosacks in dorsal appendages where the cnidocysts of the prey are stored and used for defence.

The paraphyly of the Dendronotoidea is partly consistent with conclusions based on morphological features (Wägele & Willan, 2000). Our molecular based results confirm the Dotidae as monophyletic and its exclusion from the Dendronotoidea, but even the other Dendronotoidea species lack support for a monophyletic taxon in the sense of Wägele (1997) and Wägele & Willan (2000). These authors discussed the following synapomorphies for uniting all other dendronotacean taxa (with the exclusion of 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 to all other Cladobranchia through long branches (Fig. 2). Long branches appear either if taxa evovle at a higher rate as is the case for *Tritonia nilsodhneri* and the genus *Doto*, or if they have been separated earlier than all other taxa from their common ancestor, thus showing higher divergence from the grundmuster of the last common ancestor (Swofford et al., 1996; Hendy & Penny, 1989).The later must be assumed for the remaining Dendronotoidea.

The molecular data confirm the paraphyly of the Arminoidea as had been concluded by morphological data (Wägele & Willan, 2000). Both results suggest different ancestry for the "Arminoidea" species. Kolb & Wägele (1998) have performed a thorough phylogenetic analysis of the family Arminidae based on morphological and histological characters. The autapomorphy that characterizes the family is the presence of marginal sacs in the lateral notum. The 18S, as well as the 16S analyses confirm the monophyly of the Arminidae.

The 18S rDNA gene resolves older speciation events in the evolution of the Nudibranchia and coincides best with the hypothesis Wägele & Willan (2000) based on morphological and histological data. The 18S rDNA gene is highly robust when using different phylogenetic methods, whereas this is not the case for *cox1*. The 16S rDNA and *cox 1* genes are described to solve phylogenetic questions on family, genus or even population level (e.g. Simon et al. 1994, Thollesson, 1999a, Reid et al., 1996, Lydeard et al., 1997, Remigio& Blair, 1997). In our analysis a higher solution on family or genus level in comparison to the 18S rDNA trees is not observed.

The data presented here contribute to our understanding of the relationships of nudibranch taxa. They confirm the monophyly of the Nudibranchia. They clearly show the evolution of two major lineages which are morphologically very different (Fig. 7). Nevertheless only the Anthobranchia/Doridoidea clade is characterized by newly derived 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 its 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). Therefore in this case, the molecular data are especially valuable in evaluating the conclusions based on morphological data. 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, feeding on Cnidaria species, dealing with stinging cells in their digestive tract and handling them in a way so that they can be used for own defence) are weakly or not supported by the molecular phylogenies based on the three markers. We conclude that a critical evaluation of both molecular and morphological data and hypotheses enrichs the understanding of phylogeny and evolution of the nudibranchia. Incongruencies between different data sets encourage to search for reliable hypotheses through analysing more taxa and molecular markers.

Acknowledgements

We are indebted to the following people for providing the specimens used in this study: Manuel Ballesteros (Barcelona), Gilianne Brodie (Townsville), Ulrike Englisch (Bochum), José Gonzalez (Santiago de Compostela), Christoph Held (Bielefeld), Wulf Kobusch (Bochum), Annette Klussmann-Kolb (Mainz), Katharina Noak (New York), Patricia Reboreda (Santiago de Compostela), Michael Schrödl (München), Christoph Schubart (Louisanna), Gabi Strieso (Bochum), Jesús Troncoso (Vigo), Victoriano Urgorri (Santiago de Compostela). Paula Mikkelsen (New York) kindly indentified the probable new species of *Smaragdinella*. This project was funded by the Deutsche Forschungsgemeinschaft (Wa 618/4).

References

Bergh, L. S. R. (1892): Malacologische Untersuchungen 18. System der Nudibranchiaten Gastropoden. In: Semper C. ed. Reisen im Archipel der Philippinen. 2. Teil. Bd. 2: 995-1165.

Boettger, C. (1955): Die Taxonomie der euthyneuren Schnecken. Zool. Anz. 18: 253-280.

Férussac, A. (1822): Tableaux Systématiques des Animaux mollusques. Suivie d'un Prodrome Générale: I-XLVII, 1-110.

Felsenstein, J. (1995): PHYLIP (phylogeny infered package) (Department of Genetics, University of Washington, Seattle) Vers. 3.5.

Foale, S. J. & Willan, R. C. (1987): Scanning and transmission electron microscop study of specialized mantle structures in dorid nudibranchs (Gastropoda: Opisthobranchia): Mar. Biol. 95(4): 547-557.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994): DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech. 3: 294-299.

Haszprunar, G. (1988): On the origin and evolution of major gastropod groups, with special reference to the Streptoneura. J. Moll. Stud. 54: 367-441.

Hendy, M. D. & Penny, D. (1989): A framework for the quantitative study of evolutionary trees. *Systematic Zoolology* **38**: 297-309.

Kolb, A. & Wägele, H. (1998): On the phylogeny of the Arminidae (Gastropoda, Opisthobranchia, Nudibranchia) with considerations of biogeography. J. Zool. Syst. Evol. Research 36: 53-64.

Kumar, S., Tamura, K. & Nei, M. (1993): MEGA (Molecular Evolutionary Genetics Analysis) (The Pennsylvania State, University Park, PA 16802) Vers. 1.0.6.

Lecointre, G., Philippe, H., Le, H. L. V. and Le Guyader, H. (1993): Species sampling has a major impact on phylogenetic inference. Mol. Phyl. Evol. 2: 205-224.

Lydeard, C., Holznagel, W. E., Garner, J., Hartfield, P. & Pierson, J. M. (1997): Molecular phylogeny of mobile river drainage basin pleurocerid snails (Caenogastropoda: Cerithioidea). *Journal of Molecular Phylogenetics and Evolution* **7**: 117-128.

Mikkelsen, P. M. (1996): The evolutionary relationships of Cephalaspidea s.l. (Gastropoda; Opisthobranchia): a phylogenetic analysis. Malacologia 37: 375-442.

Minichev, Y. S. (1970): On the origin and system of nudibranchiate molluscs (Gastropoda, Opisthobranchia): Monitore Zoologico Italiano 4: 169-182.

Nicholas, K. B. & Nicholas, H. B. G. (1997): Genedoc: a tool for editing and annotating multiple sequence alignment. Distributed by the author www.eris.com/~ ketchup/genedoc.shtml

Odhner, N. H. J. (1934): The Nudibranchiata. Nat. hist. Rep. Br.Terra Nova Exped. Zool. 7: 229-310.

Ponder, W. F. & Lindberg, D. R. (1997): Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. Zool. J. Linn. Soc. 119: 83-265.

Pelseneer, P. (1893-1894): Recherches sur divers opisthobranches. Mém. Cour. Acad. R. Sci. Belgique 53: 1-157.

Reid, D. G., Rumbak, E. & Thomas, R. H. (1996): DNA; morphology and fossils: phylogeny and evolutionary rates of the gastropod genus *Littorina. Philosophical Transactions Royal Society London Biololy* **351**: 877-895.

Remigio, E. A. & Blair, D. (1997): Molecular systematics of the freshwater snail family Lymnaeidae (Pulmonata, Basommatophora) utilising mitochondrial ribosomal DNA sequences. *Journal of Molluscan Studies* **63**: 173-185.

Schmekel, L. (1985): Aspects of evolution within the opisthobranchs. In "The Mollusca"(K. M. Wilbur, Ed.), pp. 221-267. Academic Press, London.

Simon, S., Frati, F., Beckenbach, A., Crespi B., Liu H. & Flook P. (1994): Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America. 87: 651-701.

Swofford, D. L., Olsen, G. J., Waddell, P. J. & Hillis, D.M. (1996): PAUP: phylogenetic analysis using parsimony. Version 4.0. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois

Tardy, J. (1970): Contribution à l'etude des métamorphoses chez les nudibranches. Ann. Sci. Nat. Zool. Biol. 12: 299-370.

Thollesson, M. (1999a): Phylogenetic analysis of Euthyneura (Gastropoda) by means of the 16SrRNA gene: use of a "fast" gene for "higher-level" phylogenies. Proc. R. Soc. Lond.B 266: 75-83.

Thollesson, M. (1999b): Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitochondrial 16S rRNA gene. J. Moll. Stud. 65: 335-353.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876-4882.

Tillier, S., Masselot, M., Guerdoux, J. & Tillier, A. (1994): Monophyly of major gastropod taxa tested from partial 28Sr RNA sequences with emphasis on Euthyneura and hot-vent limpets Peltospiroidea. Nautilus 108 (Suppl): 122-140.

Wägele, H. (1989): Die Gattung *Bathydoris* Bergh, 1884 (Gnathodoridacea) im phylogenetischen System der Nudibranchia (Opisthobranchia, Gastropoda): Z. zool. Syst. Evolut.-forsch. 27: 273-281.

Wägele, H. (1997): On the phylogeny of the Nudibranchia (Gastropoda: Opisthobranchia): Verh. Dtsch. Zool. Ges. 90.1: 184

Wägele, H. (1997/98): Histological investigation of some organs and specialised cellular structures in Opisthobranchia (Gastropoda) with the potential to yield phylogenetically significant characters. Zool. Anz. 236: 119-131.

Wägele, H. & Willan, R. C. (2000): On the phylogeny of the Nudibranchia. Zool. J. Linn. Soc. 130: 83-181.

Wägele, H., Brodie, G. D. & Klussmann-Kolb, A. (1999): Histological investigations on *Dendrodoris nigra* (Stimpson, 1855) (Gastropoda, Nudibranchia, Dendrodorididae): Moll. Res. 20(1): 79-94.

Winnepenninckx, B., Steiner, G., Backeljau, T. & DeWachter, R. (1998): Details of gastropod phylogeny inferred from 18Sr RNA sequences. Mol. Phylog. & Evol. 9(1): 55-63.

Wollscheid, E. & Wägele, H. (1999): Initial results on the molecular phylogeny of the Nudibranchia (Gastropoda, Opisthobranchia) based on 18S rDNA data. Mol. Phyl. Evol. 13: 215-226.

Takezaki, N., Rzhetsky, A. & Nei, M. (1995): Phylogenetic Test of the molecular clock and linearized trees. J. Mol. Biol. Evol. 12: 823-833.

Wu, C.-I. & Li, W.-H. (1985): Evidence for higher rates of nucleotide substitution in rodents than in man. Proc. Natl. Acad. Sci. USA 82: 1741-1745.

Table 1: List of species investigated with collection site and accession numbers

Species	Collection site	18S rDNA	16S rDNA	*cox1* Gen
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 AF249234	AF249780
Cadlina luteomarginata (MacFarland, 1966)	USA, North Atlantic	AJ224772	AF249231	AF249803
Chromodoris krohni (Verany, 1846)	Spain, 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 &	Spain, Mediterranean Sea			AF249821

Hancock, 1862				
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, 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 AJ224781	AF249228	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, Atlantic	AJ224783	AF249226	AF249788
Hypselodoris elegans (Cantraine, 1834)	Spain, Atlantic	AJ224779	AF249238	AF249787
Hypselodoris villafranca (Risso, 1818)	Spain, Atlantic	AJ224780	AF249237	
Jorunna tomentosa (Cuvier, 1804)	Helgoland, North Sea	AF249210		
Limacia clavigera (Müller, 1776)	Spain, Atlantic	AJ224778		

Onchidoris bilamellata (Linné, 1767)	Helgoland, North Sea	AJ224776	AF249235	
Phyllidia coelestis Bergh, 1905	Australia, Great Barrier Reef	AF249209		
Phylidiella pustulosa (Cuvier, 1804)	Australia, Great Barrier Reef	AF249208	AF249232	
Platydoris argo (Quoy & Gaimard, 1832)	Spain, Mediterranean Sea			AF249811
Plocamopherus ceylanicus (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, Atlantic	AF249204	AF249248	
Doto koenneckeri Lemche, 1976	Spain, Atlantic	AF249205	AF249249	AF249797
Doto pinnatifida (Montagu, 1804)	Spain, 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,	Spain, Atlantic	AF249200		

1983				
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, 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, North Atlantic	AF249198	AF249245	AF249790
Godiva banyulensis (Garcia & Garcia, 1985)	Spain, Mediterranean Sea			AF249782
Tergipes tergipes (Forskal, 1775)	Kattegat, North Sea	AF249197		

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		
TYLODINOIDEA				
Tylodina perversa (Gmelin, 1790)	Spain, Mediterranean Sea			AF249809
ANASPIDEA				
Aplysia depilans Bohatsch, 1761	Normandy, 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, Atlantic	AF249192	AF249254	
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	
CEPHALASPIDEA				

Haminoea cymbalum (Quoy & Gaimard 1935)	Australia, Great Barrier Reef	AF249221	AF249258	
Smaragdinella spec.	Egypt, Red Sea	AJ224789	AF249257	AF249806
PULMONATA				
Cepaea nemoralis Linné, 1758	Germany, Bielefeld	AJ224921	AF249259	
GENBANK:				
ANASPIDEA:				
Aplysia spec.		X94268		
„PROSOBRANCHIA“:				
Littorina littorea (Linné, 1758)		X91970		
Littorina obtusata (Linné, 1758)		X94274		

Figure legends:

Fig. 1: Phylogeny of the Opisthobranchia (combination of results of Mikkelsen, 1996 and Wägele and Willan, 2000)

Fig. 2: 18S rDNA, Neighbour-Joining tree (Kimura-2-parameter, with pairwise deletion of gaps, transitions and transversions included): Shading signifies the four major taxa described by Odhner 1934. Subordinated taxa on family-level are indicated with brackets in the figure.

Fig. 3: 18S rDNA, maximum parsimony consensus tree (heuristic search, nearest neighbour interchange) with bootstrap values (1000 replicates, 50% majority-rule consensus tree) of parsimony analysis. Only those groups with a bootstrap value higher than 50 are indicated with the value. CI: 0.58, HI: 0.42, RI: 0.79, tree length 3651 steps, shortest tree out of 1080.

Fig. 4: 16S rDNA, Neighbour-Joining tree (Kimura-2-parameter, with pairwise deletion of gaps, transitions and transversions included): Bootstrap values of the parsimony analysis with scores higher then 50 are indicated.

Fig. 5: 16S rDNA, Maximum Likelihood tree, transition/transversion ratio of 1; input of sequences ordered, Ln Likelihood = -7584.39485.

Fig. 6: *cox1*, Parsimony tree using PROTPARS (Phylip) based on amino acid sequences.

Fig. 7: Maximum parsimony tree (heuristic search, tree bisection reconnection hier musst Du die Werte nochmal nachschauen, die habe ich nicht) and bootstrap values of combined analysis with those species included, where information on all three markers is available. The inserts show representatives of the major lineages (on top: *Austrodoris kerguelenensis*, on bottom: *Flabellina pedata*): The numbers indicate the clades with

following apomorphies discussed by Wägele and Willan (2000): 1 **Nudibranchia**: rhinophores solid, absence (through loss) of the shell, pericardial complex orientated longitudinally, presence of specialized vacuolated epithelium. 2 **Doridoidea**: oesophagus without any cuticular lining, triaulic reproductive system, blood gland situated next to genital system or on top of cerebro-pleural complex, presence of gill glands. 3 **Cladobranchia**: absence (through loss) of the primary gills (ctenidium), aliform jaws, absence (through loss) of the bursa copulatrix, absence (through loss) of the blood gland.