Molecular Phylogenetics and Evolution
Vol. 15, No. 1, April, pp. 41–58, 2000
doi:10.1006/mpev.1999.0736, available online at http://www.idealibrary.com on IDEAL®

Molecular Systematics of the Order Anaspidea Based on Mitochondrial DNA Sequence (12S, 16S, and COI)[1]

Mónica Medina[2] and Patrick J. Walsh

Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149

Received February 12, 1999; revised June 21, 1999

Fragments from three mitochondrial genes (12S, 16S, and COI) were sequenced to reconstruct a molecular phylogeny of the opisthobranch order Anaspidea. The molecular phylogeny supports the placement of the genus *Akera*, a taxon previously regarded by some authors as a cephalaspidean, within the Anaspidea. Incongruence between the molecular data and the classifications based on morphology suggests that some of the taxonomic characters (i.e., shell, parapodia fusion) traditionally used for the classification of sea hares must be reevaluated, since they may be homoplastic. The ancestral nature of *Notarchus* based on the molecular evidence suggests that homoplasy may be an explanation for the morphological resemblance of this species to the more derived sea hares with highly fused parapodia and concentrated nerve ganglia. Finally, examples are given of how comparative studies of the evolution of learning mechanisms in the anaspidean clade will benefit from the phylogenetic hypothesis presented in this paper. © 2000 Academic Press

INTRODUCTION

Recent developments in the field of systematics, that take into account the evolutionary history of the traits under study (i.e., ancestral vs derived conditions), have allowed for more careful and explicit phylogenetic analyses (Hennig, 1966). In some cases, morphological characters traditionally used for classification are of questionable significance for resolving phylogenies because they are not homologous, thus obscuring phylogenetic associations between taxa. Previous attempts to determine the phylogenetic relationships of the opisthobranchs based on analysis of morphological characters have been hampered by extensive homoplasy (Gosliner, 1981, 1991; Mikkelsen, 1993). Gosliner (1991) presented comprehensive evidence of homoplasy for the whole subclass based primarily on reduction or loss of characters, and Mikkelsen (1993) presented evidence of morphological homoplasy, focusing on the relationships within the primitive Cephalaspidea. Examples of traits that have been reduced or lost many times independently are the shell, the operculum, the ctenidum, the radula, and the gizzard plates. Euthyneury (or the untwisting of the lateral nerve cords) has evolved several times from streptoneurous (or twisted) ancestors as a consequence of detorsion, i.e., the most recent Anaspidea from the primitive streptoneuran *Akera* (Gosliner, 1981, 1991). Thus, traditional opisthobranch taxonomists are faced with many homoplastic traits that are difficult to interpret for phylogenetic reconstruction.

The Anaspidea is one of the smallest opisthobranch orders. It has, however, been a group of interest not only to taxonomists (Eales, 1944; Beeman, 1968; Bebbington, 1974, 1977; Marcus, 1972) but to scientists from other disciplines of biology. Neurobiologists have chosen sea hares of the genus *Aplysia* as model organisms due the ease of following synaptical pathways through their large neuronal system (Kandel, 1979). Recently, comparative studies of related neurological and behavioral traits within the Anaspidea have appeared in the literature (Nambu and Scheller, 1986; Wright *et al.*, 1996). Ecologists have used sea hares to study chemical defense mechanisms (Avila, 1995; Pennings and Paul, 1993; Pennings, 1994; Nolen *et al.*, 1996), feeding behavior (Carefoot, 1987; Pennings and Paul, 1992), and physiological adaptation (Bedford and Lutz, 1992; Carefoot, 1991).

The order has traditionally been composed of 9 or 10 genera (Boss, 1982; Eales, 1944; Marcus, 1972; Pruvot-Fol, 1954), but relationships among these genera are unclear. The taxonomic classifications available at present for the Anaspidea have been based upon few characters, without taking into account ancestral or derived conditions (Eales, 1944; Marcus, 1972). An exception is the partial phylogeny developed by Wright *et al.* (1996). Some major taxa, however, were not

[1] The sequence data reported here have been deposited in the GenBank database under the Accession Nos. AF156109–AF156156.

[2] To whom correspondence should be addressed at present address: Josephine Bay Paul Center for Molecular Biology and Evolution, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543-1015. Fax: (508) 457-4727. E-mail: medina@evol5.mbl.edu.

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included in their analysis. The two most common taxonomic classifications are presented below. In one classification, the Anaspidea has one family (Table 1), the Aplysiidae, consisting of five subfamilies (Eales, 1944; Beeman, 1968; Boss, 1982; Willan, 1998). The other classification, based on the length of the visceral loop (Pruvot-Fol, 1933, 1954; Marcus, 1972), places the various genera into two suborders with one family each (Table 2). The suborder Longicommissurata presents long visceral nerve cords and the suborder Brevicommissurata exhibits short visceral nerve cords. Burn (1989) also used the length of the visceral loop as the diagnostic character for separating taxa. His classification is the same as that of Marcus (1972), except that he uses two subfamilies (Aplysiinae and Notarchinae) as taxonomic ranks instead of the suborders Longicommissurata and Brevicommissurata. Note the absence of *Akera* from one of these classifications (discussed below). The genus *Syphonota* may be another *Aplysia* species (Gosliner, pers. comm.; Willans, pers. comm. in Carefoot, 1987).

The systematic placement of the Akeridae has been controversial due to its streptoneurous appearance and other plesiomorphic features. Some workers place *Akera* (Marcus, 1972; Thompson, 1976; Schmekel, 1985; Burn, 1989) within the Cephalaspidea (the most primitive opisthobranch order) based on the possession of a nontentaculate cephalic shield, a large external shell, and free parapodia, in contrast to the Anaspidea, which have tentacles and rhinophores, as well as different degrees of parapodial fusion. Other workers (Morton and Holme, 1955; Pruvot-Fol, 1954; Ghiselin, 1965; Beeman, 1968; Boss, 1982; Gosliner, 1991) suggest that the Akeridae are better placed in the Anaspidea because they appear to be an intermediate step between the cephalaspidean ancestor and the rest of the sea hares. Morton and Holme (1955) and Gosliner (1991) argued that the authors that place *Akera* with the cephalaspideans based their conclusions on plesiomorphies shared between *Akera* and the Cephalaspidea as opposed to apomorphic characters. Supporters of the placement of *Akera* in the Anaspidea (Morton and Holme, 1955; Ghiselin, 1965; Gosliner, 1991) present several synapomorphies shared by *Akera* and the Anaspidea, from the reproductive system, defensive glands, radula, gizzard, and the central nervous system. Mikkelsen (1996) included the anaspidean taxa *Akera* and *Aplysia* in her study of primitive Cephalaspidea. She concluded that the Anaspidea is a monophyletic clade supported by two autapomorphic traits of the digestive system (presence of a secondary gizzard and a cecum extended from the stomach). Williams (1975) also presents the fusion of the right parietal ganglion with the supraintestinal ganglion as a synapomorphy for *Akera* and the remaining sea hares.

TABLE 1

Taxonomic Classification of the Order Anaspidea Proposed by Eales (1944), Modified by Boss (1982)

Family Akeridae	*Akera*
Family Aplysiidae	
Subfamily Aplysiinae	*Aplysia* Linnaeus 1767
	[a]*Syphonota* Adams & Reeve 1850
Subfamily Dolabellinae	*Dolabella* Lamarck 1801
Subfamily Notharchinae	*Notarchus* Cuvier 1817
	Stylocheilus Gould 1852
	[b]*Bursatella* Blainville 1817
Subfamily Dolabriferinae	*Dolabrifera* Gray 1847
	Petalifera Gray 1847
	Phyllaplysia P. Fischer 1872

[a] Synonym of *Paraplysia* (Eales, 1944).
[b] Synonymized with *Barnardaclesia* by Gosliner (1987).

TABLE 2

Anaspidean Classification Proposed by Marcus (1972), Modified from Pruvot-Fol (1954)

Suborder Longicommissurata	
Family Aplysiidae	
Subfamily Aplysiinae	*Aplysia*
	Syphonota
Subfamily Dolabellinae	*Dolabella*
Suborder Brevicommisurata	
Family Notharchidae	
Subfamily Notharchinae	*Notarchus*
Subfamily Dolabriferinae	*Stylocheilus*
	Burstella
	Dolabrifera
	Petalifera
	Phyllaplysia

Fossil Record

The outgroup taxa chosen for this study, *Bulla* and *Haminoea*, have a distinct external shell. As a consequence of strong shell calcification they have a fairly reliable fossil record. These bulloid cephalaspideans date back to the beginning of the Jurassic (200 million years ago [Wenz, 1938; Tracey *et al.*, 1993]). There is paleontological evidence that *Akera* appeared in the mid-Jurassic (165 million years ago [Tracey *et al.*, 1993]). The oldest *Aplysia* fossils date back to the Miocene (25 million years ago [Tracey *et al.*, 1993]). There is also shell evidence that the genus *Floribella*, a fossil species closely related to *Dolabella*, has been present in the fossil record since the early Miocene (25 million years ago [Geiger and Jung, 1996]). The scattered shell fossils of *Dolabrifera* have been found only from early Pleistocene deposits (1.6 million years ago [Wenz, 1938]). No thorough taxonomic revision has been conducted on these fossils because the opisthobranch fossil record is incomplete as a result of shell reduction and poor fossilization (Mikkelsen, 1996). Consequently, the paleontological record of this group is poorly known. Available fossil dates must be considered as provisional and potentially reflecting a large

margin of error. For instance, based on amino acid sequence divergence for the egg-laying hormone gene, using fossil evidence, Nambu and Scheller (1986) place the appearance of the most primitive member of the genus *Aplysia* (*A. parvula*) in the Cretaceous (140 million years ago). Thus, the genus *Aplysia* may have diverged shortly after *Akera* diverged from their most common ancestor.

Molecular Markers

Because of the high levels of morphological homoplasy and the tendency toward reduction of many of the traits traditionally used by molluscan taxonomists, additional characters must be used to resolve phylogenetic relationships within the opisthobranch clade. Molecular markers are suitable for this purpose because they provide a large number of characters that can be used in phylogenetic analyses. There are many options of molecular markers that are informative at different systematic levels. Mitochondrial DNA (mtDNA) is a molecule that has proven to carry phylogenetic signal at different taxonomic levels (Moritz *et al.*, 1987). MtDNA has been widely used for phylogenetic studies because it is maternally inherited and nonrecombining and has higher rates of evolution than nuclear genes in many taxa. Perhaps most importantly, mtDNA has an array of genes that can be useful at different phylogenetic levels (Brown, 1985; Avise *et al.*, 1987; Moritz *et al.*, 1987; Simon *et al.*, 1994). These include the ribosomal genes (12S and 16S), which are among the most conserved regions in the mitochondrial genome. Because mitochondrial ribosomal genes (rRNA) evolve at faster rates than their nuclear homologs (Mindell and Honeycutt, 1990; Hillis and Dixon, 1991; Simon *et al.*, 1994), these genes have been used primarily at lower taxonomic levels, from genus to population level (Avise *et al.*, 1987; Moritz *et al.*, 1987; Simon *et al.*, 1994). It has been suggested, however, that mitochondrial rRNA genes can be used to infer phylogenetic relationships to 300 million years old (Mindell and Honeycutt, 1990) or 65 million years old (Hillis and Dixon, 1991). The radiation of characiform fishes was used as an empirical test of the efficiency of these genes for accurately resolving phylogenetic associations. This study suggested that mitochondrial rRNA markers are reliable for divergence times to 100 million years old (Ortí and Meyer, 1997). The earliest reliably identified *Akera* shell fossils are mid-Jurassic (165 million years ago [Tracey *et al.*, 1993]), but the earliest report of the remaining primitive sea hares (*Aplysia* and *Dolabella*) is Miocene in age (25 million years ago [Tracey *et al.*, 1993]). Thus, the window of application proposed by Ortí and Meyer (1997) embraces the fossil appearance of most anaspidean taxa with the exception of the two cephalaspidean outgroup taxa. Although there are problems with using fossil evidence to date the appearance of the taxa in this study, the mitochondrial rRNA genes appeared to be appropriate markers for resolving phylogenetic relationships within this clade.

The cytochrome oxidase subunit I gene (COI) has been reported as one of the most conserved protein-encoding genes in the mitochondrial genome (Brown, 1985). Though third-codon positions are known to saturate quickly, first- and second-codon positions show intermediate-level resolution (family to genus level comparisons [Folmer *et al.*, 1994]). It has been suggested that, at even higher levels (class and phylum), the inferred amino acid sequences can be used to assess phylogenetic relationships (Folmer *et al.*, 1994). Thus, the COI gene was chosen for this study because of this potential to be phylogenetically informative at intermediate and lower taxonomic levels, possibly complementing the rRNA sequences where these regions could lack resolution.

The purpose of this research was initially to reconstruct anaspidean phylogeny based on mtDNA sequence data, then to compare it to the morphological evidence, and last to map the evolution of two behavioral traits (swimming and inking) onto the molecular phylogeny.

MATERIALS AND METHODS

Samples and DNA Isolation

Most of the samples utilized in the present study were provided by opisthobranch specialists from worldwide museums and laboratories (Table 3). Some of the samples were frozen and some were collected alive and preserved in a solution that inhibits DNA degradation (DMSO 20%, 250 mM EDTA, NaCl saturated) (Seutin *et al.*, 1991).

Total DNA was isolated by standard SDS/Proteinase K digestion (Sambrook *et al.*, 1989). Tissue was gently homogenized in an Eppendorf tube in 700 µl of 1× NET buffer (150 mM NaCl, 10 mM Tris–HCl [pH 8.0], 10 mM EDTA), with 1% SDS and 100 µg/ml proteinase K, and incubated at 65°C for 1–2 h. Nucleic acids were isolated by successive phenol:chloroform (3:1) extractions repeated until the interface was clear, followed by a chloroform:isoamyl alcohol (24:1) extraction and precipitation by the addition of Na–acetate to 300 mM and 2.5–3 volumes of absolute ethanol. Genomic DNA was resuspended in TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA) and RNase (50 µg/µl) treated at 37°C, followed by a chloroform:isoamyl (24:1) extraction and ethanol precipitation. The DNA samples were resuspended in TE buffer.

PCR Amplification and Sequencing

Amplifications were performed in 25 µl of a solution containing approximately 50 ng of DNA, 1× PCR buffer, 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.5 µM each primer, and 1.25 units of *Taq* polymerase (Perkin–Elmer/Cetus). After an initial denaturation step of 2

TABLE 3

Sample List, Distribution Range, Sampling Localities, Preservation Method, and Collector

Species	Preserv.	Distribution	Locality	Collector
Bulla gouldiana Pilsbry 1893	Frozen	Eastern Pacific	Venice, California	Marinus Marine
Haminoea virescens Sowerby 1833	Frozen	Eastern Pacific	Venice, California	Marinus Marine
Akera bullata Muller 1776	Ethanol	North E. Atlantic	Algoleran, Sweden	J. M. Turbeville
Aplysia cervina Dall & Simpson 1901	Frozen	Western Atlantic	Gulf of Mexico, Texas	Ned Strenth
Aplysia punctata Cuvier 1803	Ethanol	Circumtropical	Spain	Jesús Ortea
Bursatella leachi Blainville 1817	Frozen	Circumtropical	Florida Bay, Florida	Tom Capo
Dolabella auricularia Solander 1786	DMSO	Indo-Pacific	Guam	Steve Pennings
Dolabella auricularia Solander 1786	DMSO	Indo-Pacific	Gulf of California, Mexico	Mónica Medina
Dolabrifera dolabrifera Cuvier 1817	DMSO	Circumtropical	Guam	Steve Pennings
Notarchus indicus Schweigger 1820	Ethanol	Indo-Pacific	Ryukui Is., Okinawa	Terry Gosliner
Notarchus indicus Schweigger 1820	DMSO	Indo-Pacific	Batangas, Philippines	Terry Gosliner
Petalifera ramosa Baba 1959	Ethanol	Circumtropical	Canary Islands	Jesús Ortea
Petalifera ramosa Baba 1959	DMSO	Circumtropical	Batangas, Philippines	Terry Gosliner
Phyllaplysia sp Gosliner 1995	DMSO	Philippines	Mindoro, Philippines	Terry Gosliner
Phyllaplysia taylori Dall 1900	DMSO	North E. Pacific	Friday Harbor, Washington	Kadee Lawrence
Stylocheilus longicauda Quoy & Gaymard 1824	DMSO	Circumtropical	Okinawa, Japan	Steve Pennings

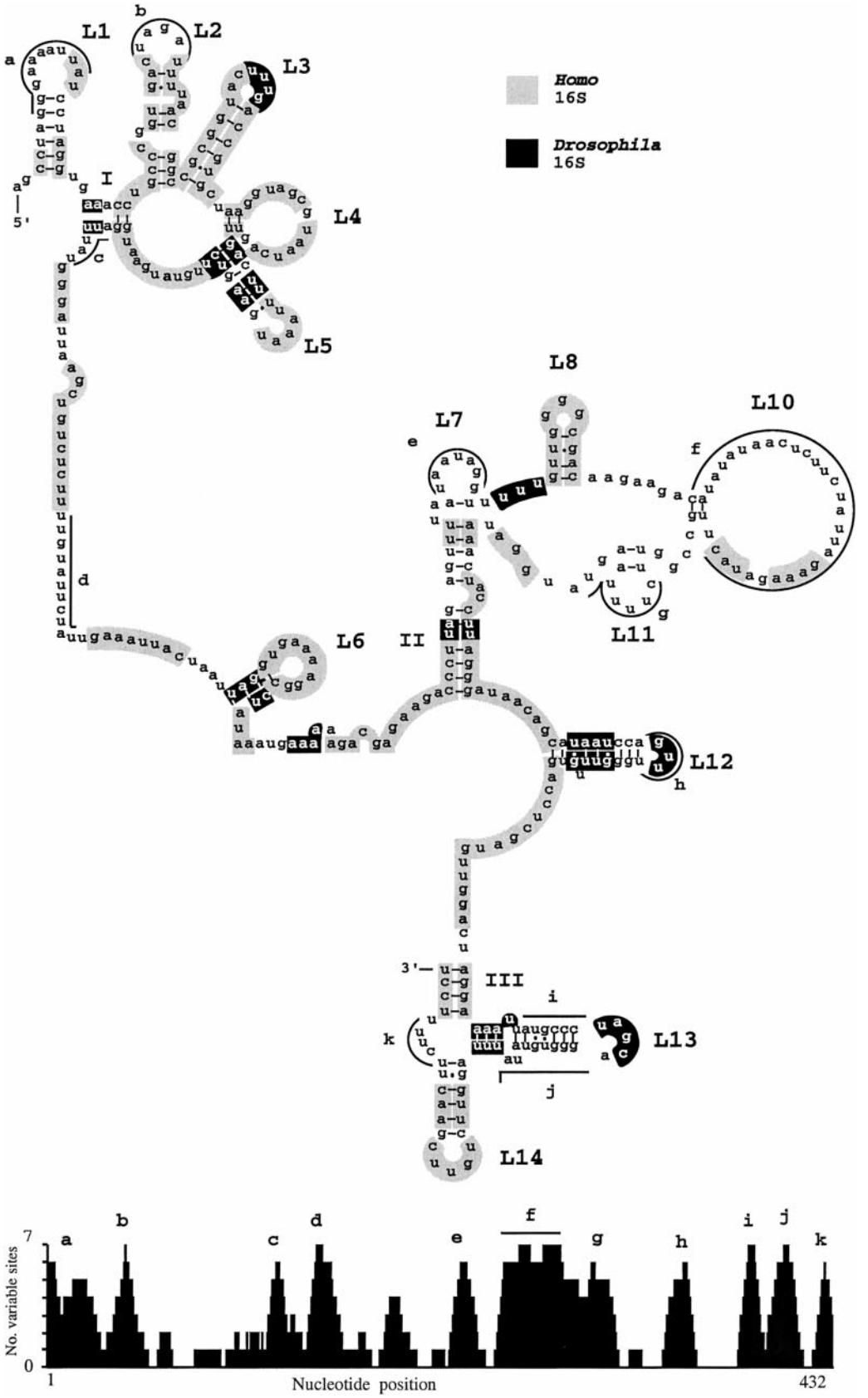
min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 45 or 50°C (45°C for 12S and 50°C for 16S and COI), and 30 s at 72°C were performed, followed by a final extension step of 5 min at 72°C. The primers used were the universal primers for the mitochondrial small ribosomal subunit (12S), 12SA-L (5′-AAACTGGGATTAGATACCCCACTAT-3′) and 12SSB-H (5′-GAGGGTGACGGGCGGTGTGT-3′), and for the large subunit (16S), 16sar-L (5′-CGCCTGTTTATCAAAAACAT-3′) and 16sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3′), developed by Palumbi *et al.* (1991). The primers used for the mitochondrial cytochrome *c* oxidase subunit I fragment were the universal primers LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) developed by Folmer *et al.* (1994).

Double-stranded PCR products were purified with the Gene Clean Kit (Bio 101). 12S and 16S primers were end-labeled with γ-^{33}P (NEN Dupont) and samples were cycle-sequenced with both primers using the Δ*Taq* sequencing kit (United States Biochemical). Samples were run in a 6% denaturing (50% w/v urea) polyacrylamide gel (19:1 acrylamide to bis-acrylamide ratio). The COI fragments were cloned into a TA vector (Invitrogen) and clones were sequenced using the Thermo Sequenase cycle-sequencing kit (Amersham) with dye-labeled universal M13 forward and reverse primers in a LiCor automated sequencer.

Sequence Alignment

The COI sequences were easily aligned by eye. The 16S and 12S sequences were aligned using the default settings of the Clustal V algorithm (Higgins and Sharp, 1988) in the multiple alignment routine of the Dnastar program (version 3.06) for Macintosh computers. Subsequently, the alignment was improved with the use of secondary structure models because stem and loop regions were easily identified. Structural homology allowed a more reliable assessment of sequence homology. A composite alignment including the three fragments can be downloaded from the University of Miami NIH–*Aplysia* Resource Facility web site (www.rsmas.miami.edu/groups/sea-hares/); regions excluded from the final analysis are marked by an asterisk below them. Regions of ambiguous alignment excluded from the final data set were present in the two ribosomal genes and were found only in loop regions. The criterion used for the exclusion of regions of ambiguous alignment was the following: in places in which more than two gaps were needed, all sites involving gaps were excluded. There were two exceptions to this rule: the entire loop regions were excluded in helix L10 of 16S (Fig. 1) and in helix 31b of 12S (Figure 2). The whole loop was excluded in both cases because large indels ($>$9) and completely different nucleotide sequences in some taxa prevented a reliable alignment, and the entire helix 42 (both stem and loop regions) of 12S was

FIG. 1. 16S Structural model for *Aplysia cervina*. Highlighted regions represent homology with the human and fruitfly mitochondrial large subunit molecule. Loop numbering follows Horovitz and Meyer (1995). Roman numbers were assigned in this study. The differences between the structural templates are discussed in the text. Lower graph represents a sliding window analysis of the 16S molecule for all the sequences including the outgroup taxa. The vertical axis represents the variable sites in a sliding window of seven nucleotides. The horizontal axis represents the position in the multiple alignment. Lower case letters (a–k) indicate the most variable regions in the structural model.


Homo
16S
Drosophila
16S
L1
L2
L3
L4
L5
L6
L7
L8
L10
L11
L12
L13
L14
I
II
III
5'
3'
No. variable sites
Nucleotide position
1
432
0
7

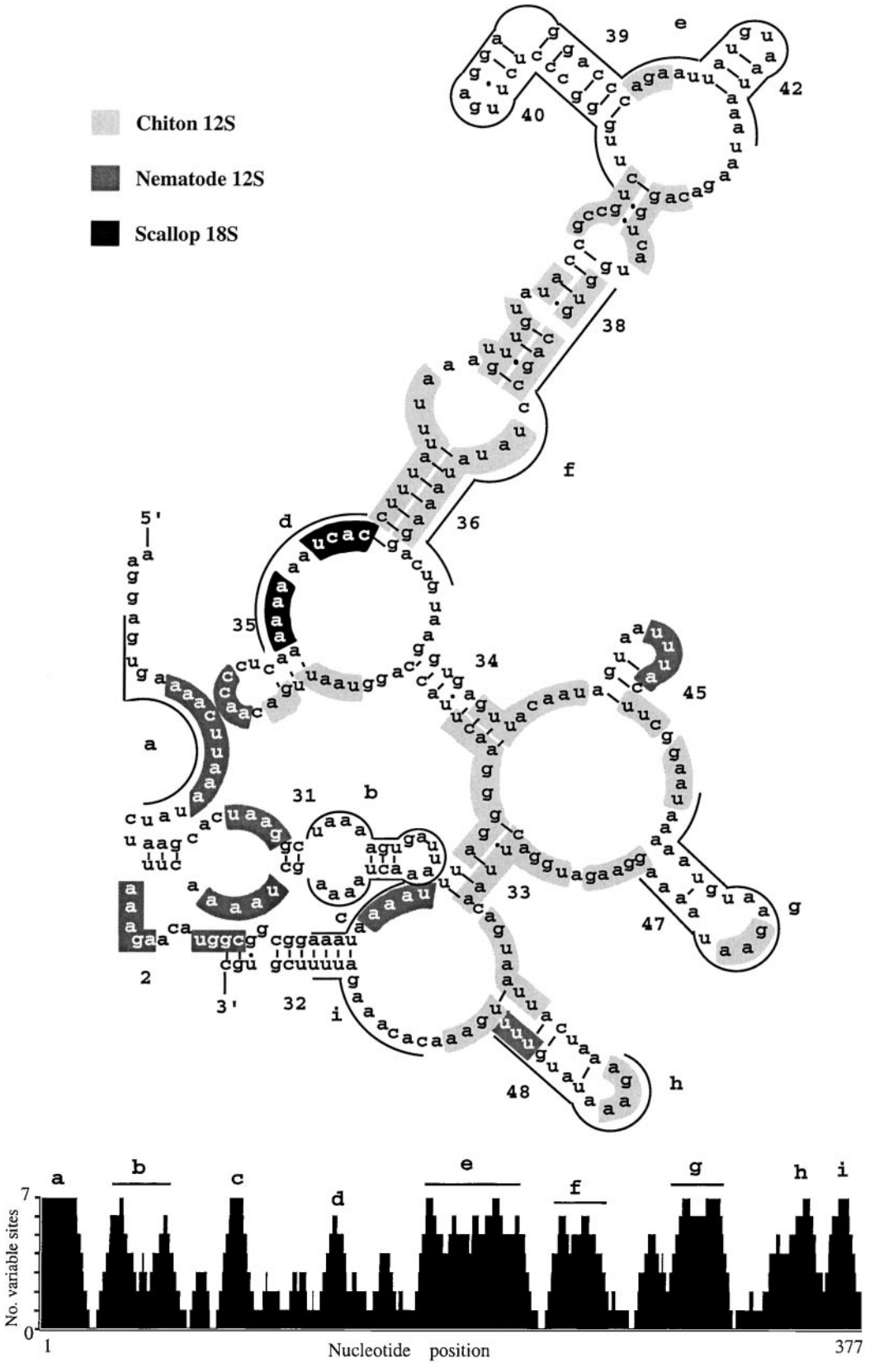


FIG. 2. 12S Structural model for *Aplysia cervina*. Highlighted regions represent homology with the chiton and nematode mitochondrial large subunit molecule and the scallop nuclear large subunit. Helix numbering follows Van der Peer *et al.* (1994). The differences between the structural templates are discussed in the text. Lower graph represents a sliding window analysis of the 12S molecule for all the sequences including the outgroup taxa. The vertical axis represents the variable sites in a sliding window of seven nucleotides. The horizontal axis represents the position in the multiple alignment. Lower case letters (a–i) indicate the most variable regions in the structural model.

excluded for the same reasons state above. The COI fragment was translated into amino acid (a.a.) data with the "invertebrate" mitochondrial genetic code in the Dnastar program, and this alignment can also be found at the NIH–*Aplysia* web site.

The use of secondary structural models can improve computer-generated alignments because stem, loop, and bulge regions can be identified (Vawter and Brown, 1993). This understanding of the secondary structure, in turn, permits a more accurate determination of homologous characters for phylogenetic analysis (Kjer, 1995). To obtain a secondary structure model for all the anaspidean taxa, both large and small subunit sequences were compared to the models developed for *Aplysia cervina* (Figs. 1 and 2). The large subunit (16S) sequence of *Aplysia* was compared to the mitochondrial model for humans (Gutell, 1994; Horovitz and Meyer, 1995) and *Drosophila yakuba* (Gutell, 1994 [Fig. 1]). The *Aplysia* small subunit (12S) secondary structure was refined with help of the model for the third domain of the 12S mitochondrial gene presented by Hickson *et al.* (1996). The rest of the molecule was compared to the 12S model for the nematode *Caenorhabditis elegans* and the 18S model for the scallop *Placopecten magellanicus*, both available from the ribosomal RNA structure web site (Gutell, 1994 [Fig. 2]). All the new structural models for the remaining Anaspidea and the two outgroup taxa are depicted in similar figures that can be downloaded from the NIH–*Aplysia* web site. Some putative stem regions could not be confirmed for the *Aplysia* model since the complementary strands of those regions were not sequenced. The only noncanonical (non-Watson–Crick) pairing allowed throughout the entire model was the wobble G · U pair, which forms two hydrogen bonds and is virtually as stable as an A · U pair (Chastain and Tinoco, 1991). On a few occasions, in which other noncanonical pairings would be necessary to maintain a structure similar to the template, symmetrical bulges (Hickson *et al.*, 1996) were invoked instead, and each region is explained below in detail. Alignment gaps placed by the computer alignment that disrupted stem pairings were moved to contiguous loop or unpaired regions of the model, and in one occasion an indel in a stem region was identified for 12S. A sliding window analysis (7 bp—following Ortí and Meyer, 1997) over the entire sequence alignment was performed in MEGA (Kumar *et al.*, 1993) to identify the most variable regions of each ribosomal molecule.

Phylogenetic Analysis

Basic statistics (nucleotide composition, transition and transversion frequencies, number of variable and parsimony sites) were performed in MEGA (Kumar *et al.*, 1993). Parsimony and neighbor-joining (NJ) analyses were conducted in PAUP* version 4.0d64 (Swofford, 1998) and maximum-likelihood analyses were performed in Puzzle 3.1 (Strimmer and von Haeseler, 1996, 1997). The sequence data were partitioned into two data sets: the rRNA (12S and 16S) genes and the COI fragment. Subsequently, these two data sets were also partitioned. The rRNA matrix was partitioned into stem and loop regions. The COI fragment was partitioned into first- and second-codon positions, and third-codon positions. The informative COI amino acid sites were included in a combined data set with the rRNA data set for parsimony analysis. The partition homogeneity test or incongruence length difference (ILD) test (Farris *et al.*, 1995) implemented in PAUP* was used to determine whether the different data sets could be combined. The settings were 10 random stepwise additions with TBR branch swapping and 1000 randomizations. Initially, rRNA pairwise sequence divergence versus COI divergence was plotted (Fig. 3). Pairwise sequence divergence for both data sets clearly increased in correlation for ingroup comparisons, whereas for outgroup comparisons, the COI pairwise divergence values tapered off at approximately 20%. To explore further saturation of the COI sequence, observed transitions (Ts) and transversions (Tv) were plotted against sequence divergence (Fig. 4). In the rRNA data set, Ts were more frequent than Tv and both increased linearly in number with increasing sequence divergence. There was an exception for the outgroup comparisons in which Tv were more frequent. This observation is an indication of Tv covering Ts (Fig. 4a). The observed substitutions at first- and second-codon positions of the COI gene follow a scattered nonlinear relationship, with Ts more abundant than Tv (Fig. 4b). In contrast is the even more scattered, nonlinear relationship of substitutions versus sequence divergence for more ancient pairwise comparisons at third-codon-position changes (Fig. 4c), in which Ts abundance tends to approximate Tv values, indicating saturation at these sites. Two combinations of weights were used for the rRNA data set (equal weights, Tv:Ts 8:1). For the weighted parsimony analysis, the Ts:Tv weighting was calculated by averaging the Ts:Tv ratios of the pairwise comparisons within the genera with more than one sample (*Aplysia, Dolabella, Notarchus, Petalifera,* and *Phyllaplysia*). The same procedure was followed to estimate the weighting used with the stem (6:1) and loop (4:1) data sets, and the COI third positions (4:1). For parsimony analysis, gaps were treated as missing characters. In all cases, heuristic searches were performed, with 10 replicates of random stepwise addition and TBR branch swapping. Consistency index (CI) was calculated as a measure of fit between the data and the reported topologies (Kluge and Farris, 1969). In cases in which more than one tree was found, a 50% majority rule strict consensus is reported. Bootstrap analysis (50% majority rule) with 1000 pseudoreplicates (Felsenstein, 1985) in PAUP* and Decay analysis in TreeRot (Sorenson, 1996) were conducted to estimate branch

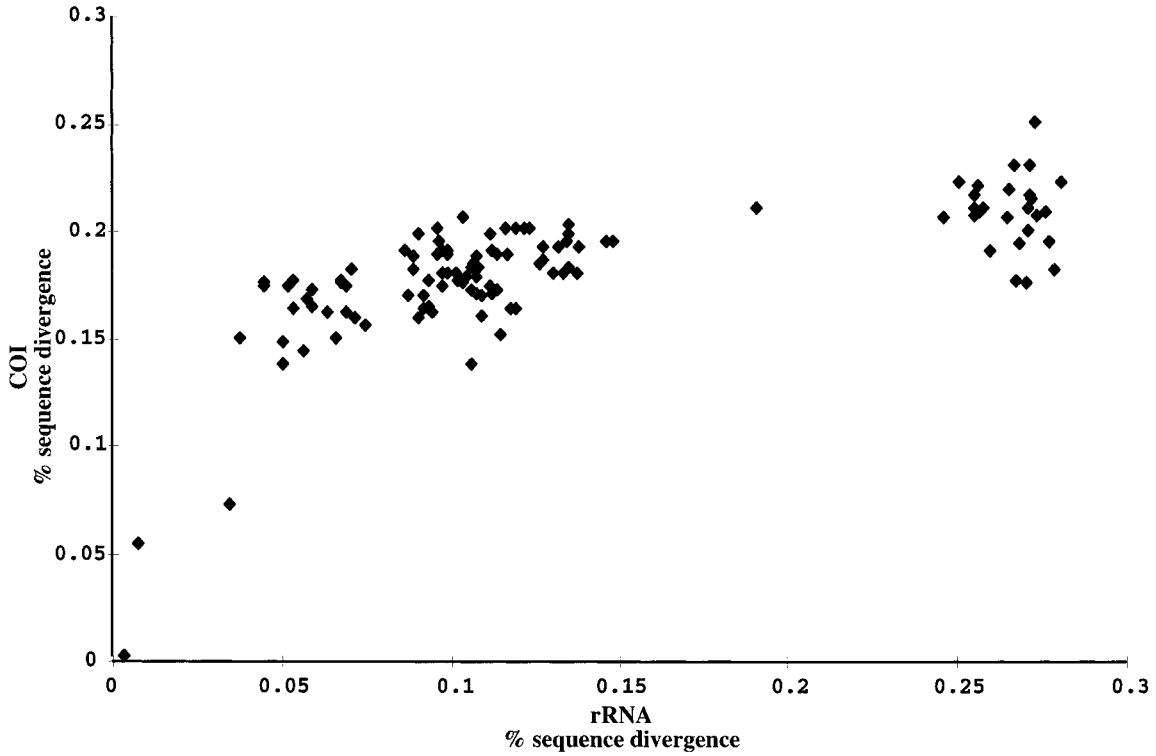


FIG. 3. Pairwise comparisons of sequence divergence (uncorrected-p) for all taxa including outgroup taxa. COI divergences on the vertical axis and rRNA (16S and 12S) divergences on the horizontal axis.

support. Neighbor-joining reconstructions were based on the Hasegawa–Kishino–Yano (HKY85) distances to accommodate multiple hits. The logDet distance was used to correct for nucleotide bias. Bootstrapping (1000 pseudoreplicates) was also performed for confidence estimation. The quartet maximum-likelihood approach implemented in Puzzle 3.1 was utilized to explore the support under the HKY85 model with 1000 quartet puzzling (QP) steps. The estimate of branch support that the QP procedure produces is similar to bootstrap support values (Strimmer and von Haeseler, 1996, 1997).

RESULTS

Mitochondrial Data

The three gene fragments comprised a total of 1372 sites, of which 564 were variable sites and 439 were parsimony informative. All three genes presented an A-T nucleotide bias (61.4% for 16S, 63.9% for 12S, and 62.6% for COI). The average A-T nucleotide bias varied within codon positions of the COI gene (56.8% for first positions, 57.5% for second positions, and 76.3% for third positions). A 658-nucleotide fragment of the COI gene was sequenced for all taxa. The average nucleotide composition on the coding strand was 23% A, 39% T, 18% C, and 20% G. In this portion of the gene, third-codon positions were the most variable sites, accounting for 78% of the overall variation, with first- and second-codon positions accounting for 19 and 3% of the variable sites, respectively. Nucleotide sequences were translated into amino acids (alignment can be downloaded from the NIH–*Aplysia* web site). Seven amino acids were common to all Anaspidea (a.a. 31, 59, 103, 104, 126, 155, and 175); a phenylalanine and a valine (a.a. 30 and 178) distinguished the genus *Phyllaplysia*. *Akera* shared one isoleucine with the outgroups (a.a. 96). *Aplysia* shared an a.a. with the outgroup (a.a. 32). *Akera* and *Notarchus* shared a leucine at position 33. The rest of the variable sites were uninformative characters.

From the 16S ribosomal gene, a fragment of approximately 432 bp was sequenced, of which 48 bp were excluded from the phylogenetic analysis. The secondary structure model for *Aplysia cervina* is depicted in Fig. 1. The average nucleotide composition of all taxa for 16S was 30% A, 31% T, 16% C, and 23% G. The alignment required two to nine indels (insertion/deletion events) per sequence (0.7–5.3% of the aligned 16S sequence length). Most indels comprised up to four nucleotides, except for a larger indel of nine nucleotides in the outgroup taxa.

Finally, a fragment of approximately 377 bp was sequenced from the 12S gene, of which 47 bp were excluded from the final analysis. The proposed secondary structure model for *Aplysia cervina* is presented in

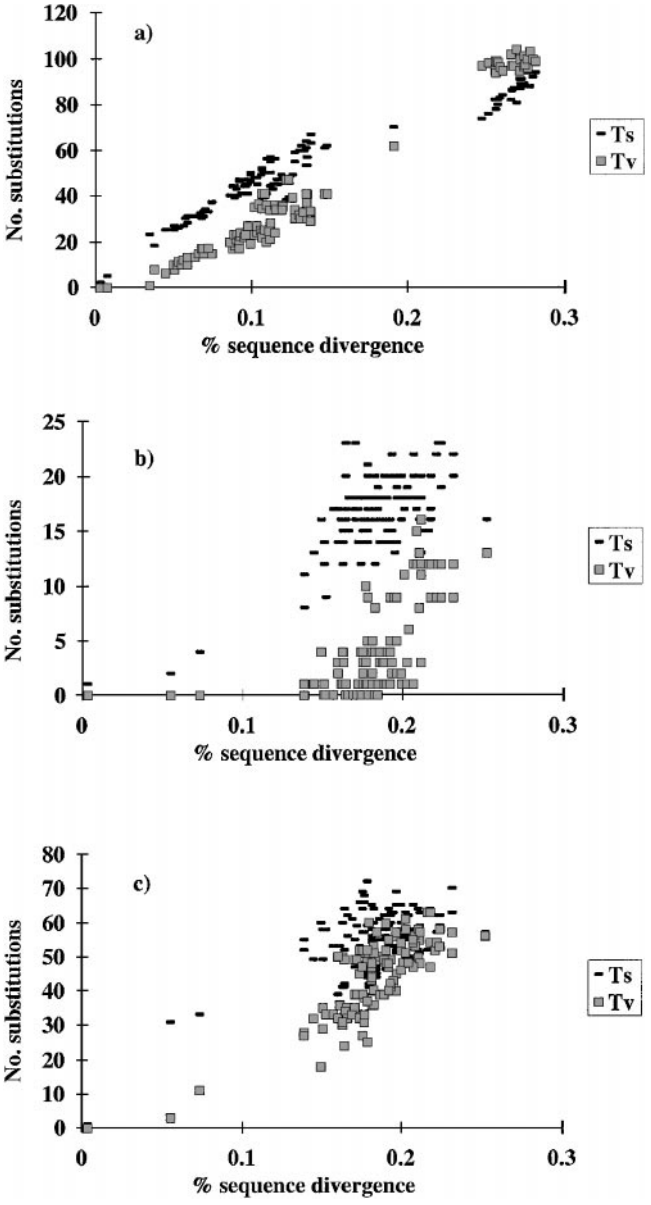


FIG. 4. Plot of observed number of transitions (Ts) and transversions (Tv) versus percentage sequence divergence (uncorrected-p). (a) rRNA data set; (b) first and second positions of COI; (c) third positions of COI.

Fig. 2. Average nucleotide composition of all taxa for 12S was 39% A, 25% T, 16% C, and 20% G. The alignment required 12–18 indels per sequence (4.7–12.4% of the aligned 12S sequence length). Indels were larger than in the 16S region, the largest being 19 nucleotides long.

The rRNA data set was separated into two structural categories, stems and loops, using the same criteria as Ortí *et al.* (1996). Only regions that were paired in the secondary structure models were considered stems. Loop regions included hairpin loops, multibranched loops, and internal loops in stem regions. Bulges and putative stem and loop regions that could not be verified due to lack of complementary sequences were excluded from this analysis. No compositional bias was observed in stems (50% A-T content), whereas loop regions showed a great A-T bias (69.8% of all nucleotides).

Sequence divergence (uncorrected-p) among taxa for the ribosomosal and the COI data sets can be downloaded from the NIH–*Aplysia* web site. For the rRNA data set, variation within the ingroup ranged from 0.3 to 14.8%, and between Anaspidea and *Bulla* and *Haminoea* it ranged from 24.6 to 28.1%. For the COI data set, these values ranged from 0.3 to 19.9% between anaspidean taxa and from 18.2 to 23.1% for outgroup comparisons.

Secondary Structure Models

The nomenclature proposed by Kjer (1995) was used to mark different structural helices on the multiple alignment (available at NIH–*Aplysia* web site). The secondary structure model for the 16S gene of *Aplysia cervina* is presented in Fig. 1 (the models for the rest of the anaspidean taxa and the outgroup can be downloaded from the NIH–*Aplysia* web site). Loop regions were identified following the numbering of Horovitz and Meyer (1995), with some stem regions additionally assigned roman numerals (I, II, III) to locate them on the multiple alignment. The model differed from the human and *Drosophila* models more noticeably in the shortening of loop regions L7 and L11. The loop region L9 from the model of Horovitz and Meyer (1995) could not be identified in the sequences in this study. The L10 loop was smaller in the outgroup taxa and the L11 stem pairings were not clearly identified in some taxa. Once the structural model was developed and the sequences were aligned, a sliding window analysis (seven nucleotides) similar to the one presented by Ortí *et al.* (1996) was followed to identify variable regions (Fig. 1). The most variable regions (identified by lowercase letters) were found in loops, in agreement with the analysis for piranhas of Ortí *et al.* (1996), except for the stem region of L13 (Fig. 1). Regions b, c, d, e, f, and g were also among the most variable in the piranha study for the 16S molecule.

The model of the 12S third domain presented by Hickson *et al.* (1996) and the 12S and 18S models for the nematode *Caenorhabditis elegans* and the scallop *Placopecten magellanicus* (Gutell, 1994) were used to reconstruct the *Aplysia cervina* 12S secondary structure (Fig. 2). The models for the rest of the anaspidean taxa and the outgroup are available at the NIH–*Aplysia* web site. Helices were numbered following Van de Peer *et al.* (1994). Helices 40, 42, 47, and 48 were the regions that differed the most in sequence composition from the template structural models; however, these structures were identified once the backbone of the molecule was reconstructed. In the loop of helix 31,

there was an indel of variable size (13–17 bp) in the two *Aplysia* sequences that was absent in the outgroup (Fig. 2). An indel of one nucleotide was also found in all ingroup taxa in helix 33. In the outgroup taxon *Bulla* the internal loop regions between helices 35 and 36 and between helices 36 and 38 present two large indels of five and four nucleotides long. A slippage event (Kjer, 1995) was necessary for helix 39 to maintain the sequence alignment in *Bulla* with the other outgroup taxon *Haminoea* (see alignment at NIH–*Aplysia* web site). Helix 42 could not be identified in the genus *Notarchus* (NIH–*Aplysia* web site). Stable symmetrical bulges (Hickson *et al.*, 1996) were necessary to maintain the structure in helices 47 and 48 (Fig. 2). Variable regions are highlighted in Fig. 2, also using a sliding window of seven nucleotides. Loop regions were again the most variable, but some stems also exhibited polymorphism. Regions b, c, e, f, and g were also among the most variable in the piranha study (Ortí *et al.*, 1996).

Phylogenetic Analysis

Based on the ILD test, the rRNA and COI data sets supported significantly different phylogenetic hypotheses ($P = 0.001$). The COI gene, though one of the most conserved protein-coding genes of the mtDNA, is thought to evolve overall at a much higher rate than the ribosomal genes. Saturation, especially at third-codon positions, was considered a possible cause of the conflicting signal. To evaluate possible saturation, pairwise sequence divergence values were plotted for the two data sets (Figs. 4a–4c). Because saturation at third-codon positions seemed to be one of the contributing factors to the conflict in phylogenetic signal between the rRNA data set and the protein-coding gene, they were excluded in a posterior analysis. In a second partition homogeneity test, the rRNA data set was run versus the COI first and second positions only ($P = 0.004$). This test also indicated conflicting signal in the two data sets; so, the data sets were analyzed separately.

The equal-weights rRNA analysis produced a single most-parsimonious tree of 619 steps with a consistency index of 0.688 (Fig. 5a). This tree differed from the traditional classifications in two ways: (1) the placement of *Notarchus* as sister taxa with *Dolabella* and basal to the rest of the taxa with short visceral loop (Brevicommissurata) and (2) *Petalifera* as sister taxa with *Bursatella* and *Stylocheilus* rather than with *Dolabrifera* and *Phyllaplysia* (see Table 1). Monophyly of the Anaspidea was supported by a 100% bootstrap value. Monophyly of the genera *Aplysia*, *Notarchus*, and *Petalifera* was also well supported with bootstrap values of 98–100% and decay indices of 6–29, except for the *Phyllaplysia* node with a relatively high support of 84% and a decay index of 3. The NJ analysis with both the HKY85 and the logDet distances produced topologies that diverged from the equal weights parsimony analysis in the placement of the taxa *Notarchus*, *Dolabella*, and *Aplysia* but bootstrap values were low for these nodes with all the analyses (Fig. 5a). The maximum-likelihood analysis performed by quartet puzzling with the HKY85 model of evolution, under the assumption of rate homogeneity, also revealed results similar to those of all the previous analyses, given that the relationships of *Aplysia*, *Dolabella*, and *Notarchus* were not clearly recovered. The branch support given by this method is also depicted in Fig. 5a. When the rRNA data set was analyzed with rate heterogeneity (gamma distribution) most branches were collapsed at the base of the tree and the support for the remaining nodes was low, except for the genus-level nodes. The weighted parsimony analysis produced a single tree in which the genus *Notarchus* is basal to the remaining taxa (Fig. 5b).

It has been suggested that rRNA stem and loop regions contain different phylogenetic signals; consequently, special care should be given to any analysis using rRNA data (Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993; Vawter and Brown, 1993; Simon *et al.*, 1994). The objective of partitioning the rRNA data set into stems and loops was to identify at what systematic level the two structures were more informative. The equal-weights parsimony analysis of the stem data set produced 5 shortest trees (TL = 141, CI = 0.816); a strict consensus tree is presented in Fig. 6a. The 6:1 weighting produced 9 most-parsimonious trees of length 346 (data not shown). The stem data set recovered the topology of deep nodes with high bootstrap support; however, some of the genus-level relationships were collapsed into polytomies with other taxa. The parsimony analysis of the loop regions produced 47 shortest trees (TL = 217, CI = 0.664); the bootstrap consensus tree is presented in Fig. 6b. When a 4:1 Tv:Ts weighting was used, 2 shortest trees were produced (TL = 514, not shown). The loop data set failed to recover the deeper nodes in the tree, but it was useful for lower-level comparisons. Both distances in the NJ analyses produced the same tree with similar bootstrap values. The NJ results were congruent with the findings of the parsimony analysis (Fig. 6b).

Initially, the COI fragment was analyzed complete with three different weightings for third-codon positions (equal weights, 4:1, and Tv only); then, third-codon positions were excluded. When equal weights were used, a single tree was produced (TL = 957, CI = 0.437), with no bootstrap support for any node. The monophyly of the ingroup was disrupted and the topology was completely different from the rRNA data set (tree not shown). *Notarchus* was the most basal taxon, and *Haminoea* appeared as sister taxa with *Akera* in a terminal node in the tree. The monophyly of the genera was maintained except for *Aplysia*, which became a grade ancestral to the *Haminoea–Akera* clade. Different weighting schemes for third positions

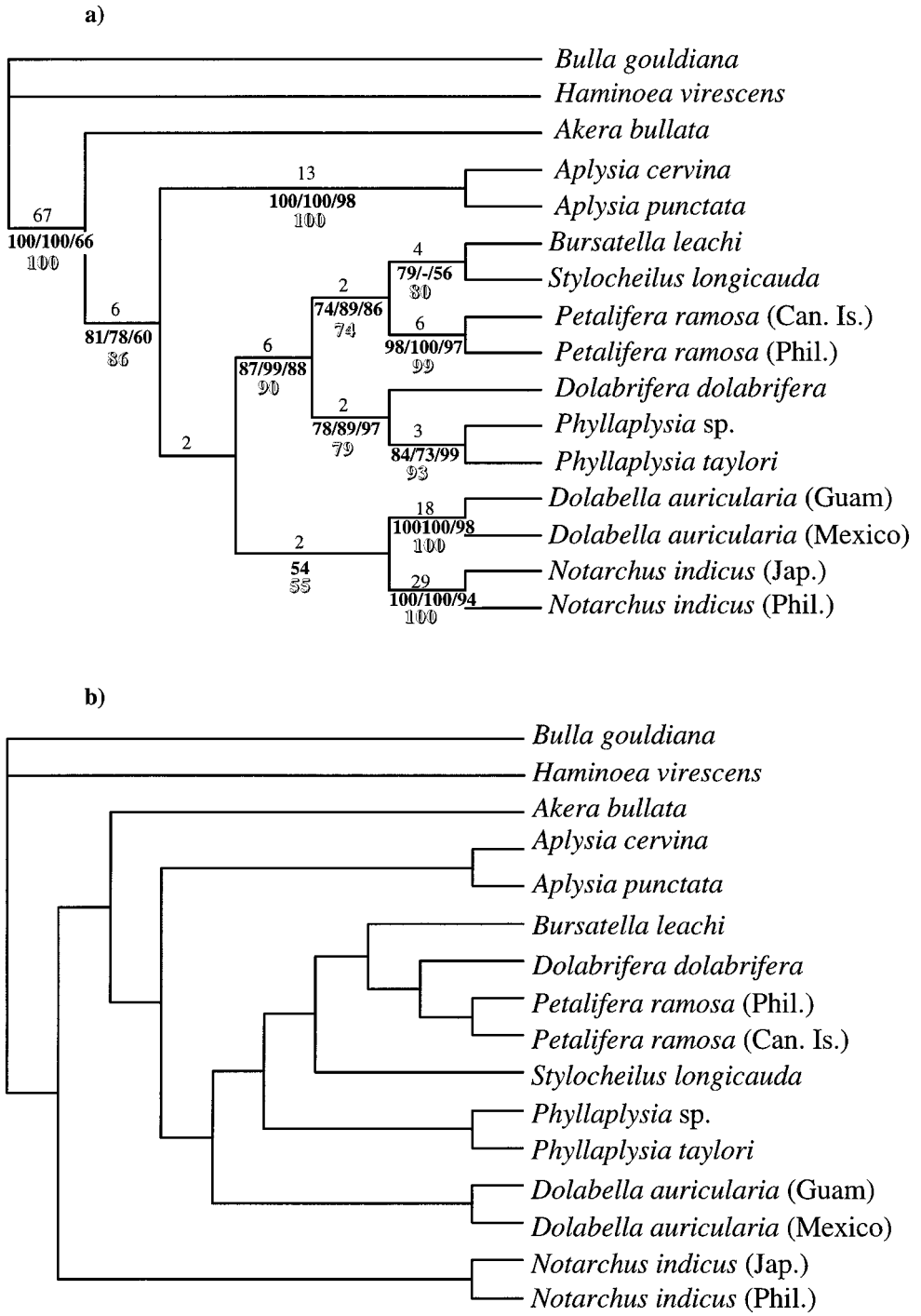


FIG. 5. (a) rRNA most-parsimonious tree produced by a heuristic search under equal weighting (TL = 619, CI = 0.688). Decay indices are depicted above each branch and bootstrap values and branch support are depicted in boldface below the branches (parsimony/neighbor-joining/quartet puzzling). In shadow are the bootstrap values for the equal weights combined analysis of COI amino acid and rRNA data. (b) Weighting of 8:1 Tv:Ts produced a single shortest tree (TL = 2314). (Can. Is., Canary Islands; Phil., Philippines; Jap., Japan).

did not improve phylogenetic signal. Two trees were produced for Tv:Ts 4:1 (TL = 1880), and there was again no bootstrap support for any node. The Tv-only analysis produced four trees (TL = 454); it also produced topologies that disrupted monophyly of the ingroup and placed *Akera* as a recently derived taxon. Because these additional weightings did not improve the resolution of the data set, no bootstrap analyses were performed. In the NJ (HKY85, logDet) analyses, a different tree was obtained, with no support for the

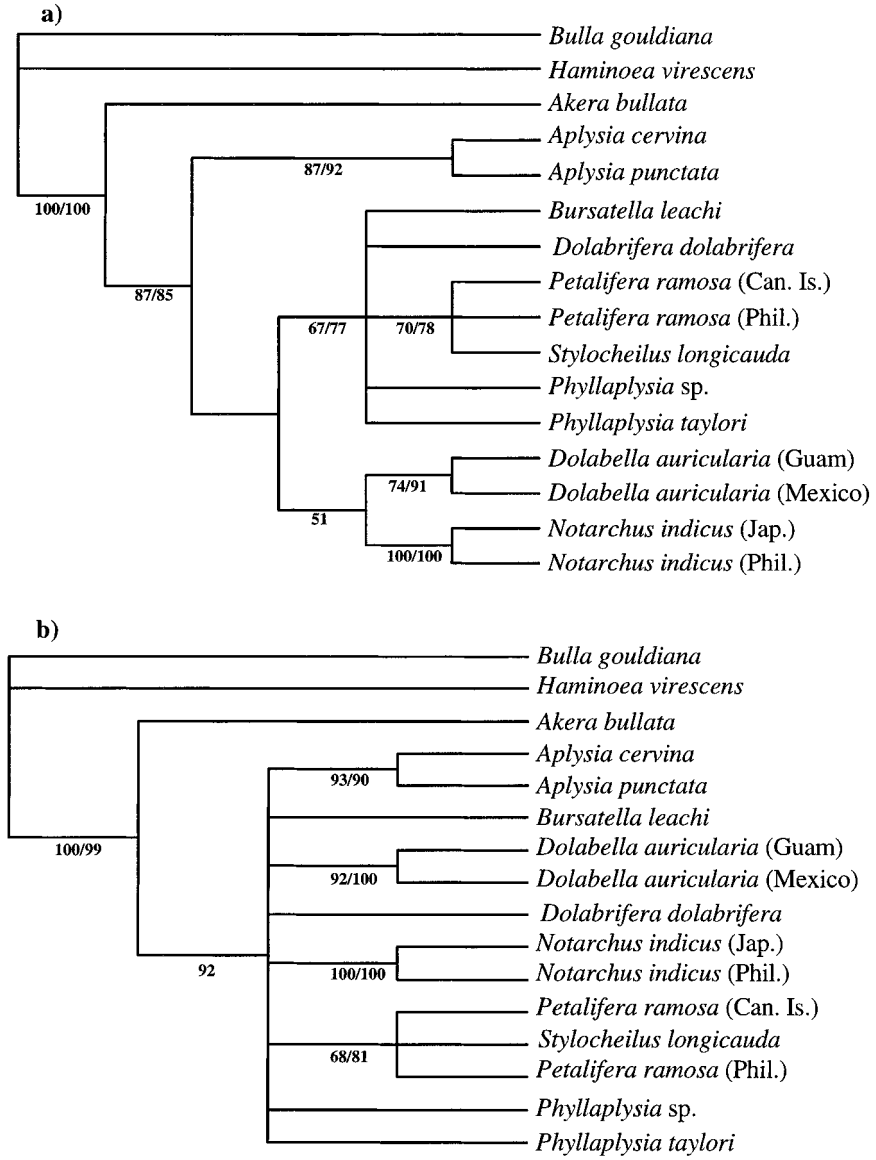


FIG. 6. (a) Strict consensus of five shortest trees with equal weighting for the stem data set (TL = 141). Parsimony and NJ bootstrap values are depicted below each branch. (b) Bootstrap consensus of 47 trees under equal weighting for the loop data set (TL = 217). Parsimony and NJ bootstrap values are depicted below branches. (Can. Is., Canary Islands; Phil., Philippines; Jap., Japan).

monophyly of Anaspidea and low or no bootstrap support for most nodes, except at the genus level. Because third-codon positions contained misleading signal, they were excluded in a subsequent analysis. An equal-weights analysis (first- and second-codon positions only) produced two most-parsimonious trees of 135 steps and a consistency index of 0.452. Even though the ingroup did show monophyly, the topology did not resemble the relationships produced by the rRNA and morphological data. First- and second-codon positions exhibited low substitution rates (Fig. 4b), which suggested no saturation at these sites. Therefore, no additional weightings were used for these positions. Results similar to those of the parsimony analysis were produced by the NJ (HKY85) analysis, but the logDet distance produced a slightly different tree. These analyses showed that the first- and second-codon positions had low signal, which was also misleading, but in this case was due to lack of informative sites.

Combined Analysis

The COI amino acid data set contained 24 variable sites, and only 12 were parsimony informative characters. These characters were also combined with the rRNA data set and an equal-weights parsimony analysis was performed. A single shortest tree was produced

(TL = 638, CI = 0.694) with the same topology as the one obtained with the rRNA data set alone. Bootstrap support was increased for most nodes (Fig. 5a).

DISCUSSION

Phylogenetic Analysis

Ribosomal genes. The rRNA data set resolved with high confidence the monophyly of the Anaspidea and the relationships of the most derived sea hares (Fig. 5a). The low support for the intermediate nodes (*Aplysia, Dolabella,* and *Notarchus*) might reflect the need for additional data or might be due to saturation of nucleotide changes. A third possibility is that there was a rapid radiation of these three lineages after divergence from *Akera.* Weighting of Tv 8:1 over Ts in the parsimony analysis did not improve phylogenetic signal and reduced support for most nodes (Fig. 5b). This weighting scheme changed the topology by placing *Notarchus* as ancestral to *Aplysia* and *Dolabella,* which would force one to invoke unlikely morphological reversals, such as (a) elongation of the visceral loop once it was concentrated around the anterior nerve ring, (b) separation of previously fused nervous ganglia, and (c) reappearance of the adult shell once it was lost. Nerve cord shortening, ganglionic fusion, and adult shell reduction and loss are common in other opisthobranch orders (Gosliner, 1991, 1994). Thus, even though these characters do exhibit high levels of homoplasy in other opisthobranch clades, the evolution of these morphological features would have to have occurred in the opposite direction in the anaspidean clade. Consequently, the equal-weights parsimony topology seems to be a better approximation to the phylogenetic relationships within the Anaspidea. This topology was also supported by the logDet NJ and the maximum-likelihood QP analysis. The assumption of rate homogeneity for the QP analysis had more phylogenetic resolution and gave results congruent with those of the other methods. Even though the maximum-likelihood analysis with rate heterogeneity is considered a more realistic model of molecular evolution, most of the phylogenetic signal was lost when this approach was used because it suggested some star-like evolution of the rRNA sequences.

Stems and loops. Only part of the rRNA data set was used in the analyses of these structures, because the structural model for some stem and loop regions could not be corroborated due to incomplete sequence data. From the stem data set we were able to produce the basal nodes in the tree with high confidence levels, but this tree lacked resolution for some of the genus-level comparisons (Fig. 6a). When equal weights were used, the loop data set contained high support for the monophyly of Anaspidea but the basal nodes were all collapsed into a polytomy (Fig. 6b). Recent nodes are better supported by the loop data set than by the stems, which is an indication of higher substitution rates in loop regions. There was some indication of homoplasy in the loop data set (CI = 0.664) compared to the stem data set (CI = 0.816). However, to account for multiple hits in both data sets, two weightings were used. For the stem data set, a 6:1 weighting was used. For the loop regions, a 4:1 weighting was used (trees not shown). Stem regions seem to have been evolving at slower rates than loop regions and have not accumulated enough changes for resolving recent divergences. Both structural domains had complementary information and the use of the complete rRNA data set had better resolution for resolving the relationships within the Anaspidea.

Cytochrome oxidase c I gene. The COI fragment used for this analysis failed in recovering with confidence both the monophyly of the Anaspidea and any of the phylogenetic relationships within the anaspidean clade (tree not shown). This is illustrated by the low consistency index (0.437) of the data set and the low bootstrap support for all nodes. Different weightings, including the different distances in the NJ analyses, did not improve phylogenetic signal because third positions were saturated (Fig. 4c). Exclusion of third positions did not enhance the consistency index (0.452) and only helped recover with relative confidence both the monophyly of the Anaspidea and some of the recent divergences. The translated amino acid sequences did not contain sufficient phylogenetically informative sites to resolve most relationships within the Anaspidea (a.a. alignment available at NIH–*Aplysia* web site). However, there were seven synapomorphic amino acid sites that also confirmed monophyly of the order and some amino acids that confirmed monophyly of the genera *Phyllaplysia, Notarchus,* and *Aplysia.* Therefore, when using nucleotide data, the COI gene is not a reliable molecular marker for ancient divergences such as the anaspidean taxa. Analysis at the nucleotide level with this gene should be restricted to genus- or species-level comparisons. In contrast, the amino acid sequences were highly conserved and little phylogenetic signal was recovered. Despite the low variability at the protein level, the fact that monophyly of the order Anaspidea was supported by seven amino acids, increasing the bootstrap confidence in a combined analysis with the rRNA genes (Fig. 5a), suggests that this gene could be useful in resolving relationships at higher systematic levels when amino acid data are used.

Taxonomic Implications of the rRNA Evidence

The concentration of the nerve ganglia in the anterior part of the head associated with the shortening of the visceral loop (Brevicommissurata) is known as cephalization (Gosliner, 1994). Cephalization is common in several opisthobranch lineages and has evolved in parallel from different ancestral taxa with long visceral cords. However, the visceral loop has been a

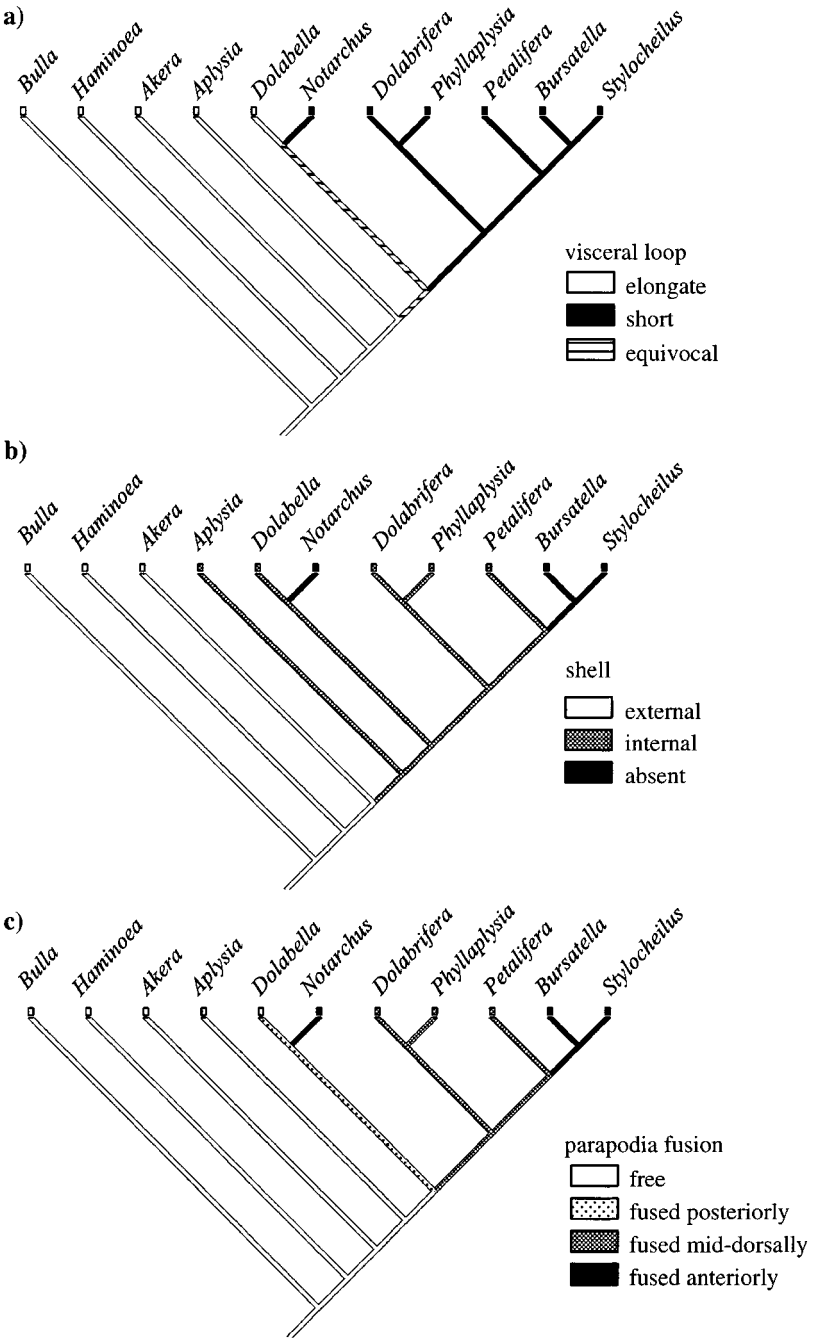


FIG. 7. Morphological characters rendered homoplastic by the molecular phylogeny (rRNA). Character tracing was performed in MacClade 3.06. Equivocal cycling was used. (a) Shortening of the visceral loop, (b) shell loss, and (c) fusion of parapodia.

key character in taxonomic revisions of the Anaspidea, and in one of the common classifications it is used to divide the order into the suborders Longicommissurata and Brevicommissurata (Pruvot-Fol, 1954; Marcus, 1972). The molecular evidence presented in this study weakly suggests an association between *Notarchus* and *Dolabella* (Fig. 5), which could be explained by two possible scenarios (Fig. 7a). (1) The most parsimonious scenario would be that *Dolabella* is ancestral to *Notarchus* and they diverged almost at the same time from their *Aplysia* ancestor. Subsequently, the *Notarchus* lineage would have given rise to the remainder of the Brevicommissurata. This hypothesis would not require one to invoke the shortening of the visceral loop to occur twice in the anaspidean clade. (2) An alternative hypothesis would be that cerebralization (Brevicommissurata) might have occurred in two separate instances in the anaspidean clade instead of only once.

Notarchus, a taxon with short visceral loop, would have evolved from a lineage that also gave rise to the primitive Longicommissurata *Dolabella. Dolabella* shows a pronounced fusion of ganglia which is also observed in *Notarchus.* This would suggest that the fusion of the nerve ring ganglia and the visceral ganglia was the initial step of cerebralization in this clade. Then, the lateral nerve cords were shortened in *Notarchus,* whereas in *Dolabella* the visceral loop remained elongate after the fusion of the anterior ganglia and the visceral ganglia. This evidence opposes the previous belief that *Notarchus* diverged from the same lineage as the rest of the Brevicommissurata in this order. The molecular evidence suggests the possible parallel evolution of cephalization, which would render inappropriate the separation of the sea hares by Pruvot-Fol (1954) into the suborders Longicommissurata and Brevicommissurata (Table 2). However, additional molecular data are needed to confirm support for the *Dolabella* and *Notarchus* clade.

Other morphological characters would require homoplastic changes if the molecular tree is preferred. Most of these characters are part of the external anatomy and tend to be diverse characters in other opisthobranch groups, as well. For example, the evolution of shell morphology in the Anaspidea, as in all opisthobranch orders, is toward shell loss. Taxonomists have used shell characters to identify the different Brevicommissurata families, but shell loss seems to have occurred in parallel in several lineages of the sea hares rather than only once (Fig. 7b). In fact, it has been shown that in the genus *Phyllaplysia* there is great plasticity in shell morphology, such that in some species there can be adult individuals with and without a shell at the same time in a population (Beeman, 1963; Marcus, 1972). Another example is the degree of parapodial fusion, which is variable within the genus *Aplysia* and possibly a plastic trait in the other anaspidean taxa. The morphological trend in sea hares is toward tight parapodial fusion, which started with the posterior fusion in *Aplysia* from the free parapodia in *Akera* and the outgroups. The subfamily Notarchinae of Eales (1944) contains the genera *Notarchus, Bursatella,* and *Stylocheilus* (Table 1). The anterior fusion of the parapodia has been interpreted as a synapomorphy of this clade. The molecular data indicate that the subfamily Notarchinae is not a monophyletic clade. Therefore, the resemblance in parapodial fusion between these three taxa might be explained by convergence of this trait in *Notarchus,* possibly due to its specialized mode of swimming (Fig. 7c [see below]).

The Evolution of Swimming

Two different types of swimming have been identified within the Anaspidea: the metachronal or "butterfly" swimming (anterior to posterior parapodial oscillations [Hamilton and Ambrose, 1975; Von der Porten and Parsons, 1982]) present in *Akera* (Morton, 1955) and some *Aplysia* species (Eales, 1960; Carefoot, 1987) and the "somersaulting" swimming, which is driven by jet propulsion (Pruvot-Fol, 1954; Martin, 1966; Willan, 1998). *Akera* tends to swim in an upward direction, whereas *Aplysia* moves horizontally in the water column and *Notarchus* swims in cycles of one or several somersaults. The topology produced by the molecular evidence seems to indicate that swimming has a phylogenetic component. Even though the swimming mechanisms in the three taxa are somewhat different, this behavior seems to have appeared in the ancestral anaspidean and then subsequently to have been lost in two instances (Fig. 8a), once in the lineage that led to *Dolabella* after splitting from *Notarchus* and again in the ancestor of the remaining Brevicommissurata (Fig. 8a). If the two swimming mechanisms are not homologous, then an alternative scenario for the evolution of this behavior would be that parapodial flapping is a plesiomorphic state and it was lost in more derived taxa, and swimming by jet propulsion evolved independently in the genus *Notarchus.* Homology of swimming mechanisms could be tested at the neurological level since the neural network that controls swimming has already been identified in *Aplysia* species (Gamkrelidze *et al.,* 1995).

The Evolution of Defensive Glands

The defensive glands of sea hares have been identified as unique features of this opisthobranch group (Gosliner, 1994). Even though the opaline and purple glands are called defensive glands, there is controversy about their function (Nolen *et al.,* 1996). For instance, it has been suggested that the purple gland has an excretory function rather than a defensive role (Chapman and Fox, 1969). The purple gland has been studied in more detail than the opaline gland, especially at the neuronal level (Carew and Kandel, 1977). Most of these studies have been performed on the model organism *Aplysia californica* under artificial stimuli, which induces inking behavior (Carew and Kandel, 1977). Inking behavior (release of purple ink) has been detected widely in other sea hares of the genus *Aplysia* (Eales, 1960) and in several sea hares of other genera (Gosliner, 1994; Kandel, 1979). The ability to release ink from the purple gland appeared after the anaspidean lineage diverged from the cephalaspidean ancestor, and it has been lost in several lineages independently (Fig. 8b). Therefore, the evolution of inking behavior does not seem to be phylogenetically constrained after appearing in the Anaspidea. It has been shown that *Aplysia* species extract the purple ink components from their seaweed diets (Nolen *et al.,* 1995); thus, the ability to ink might be correlated to the availability of red seaweed or the dietary habits of each species, rather than to an evolutionary constraint.

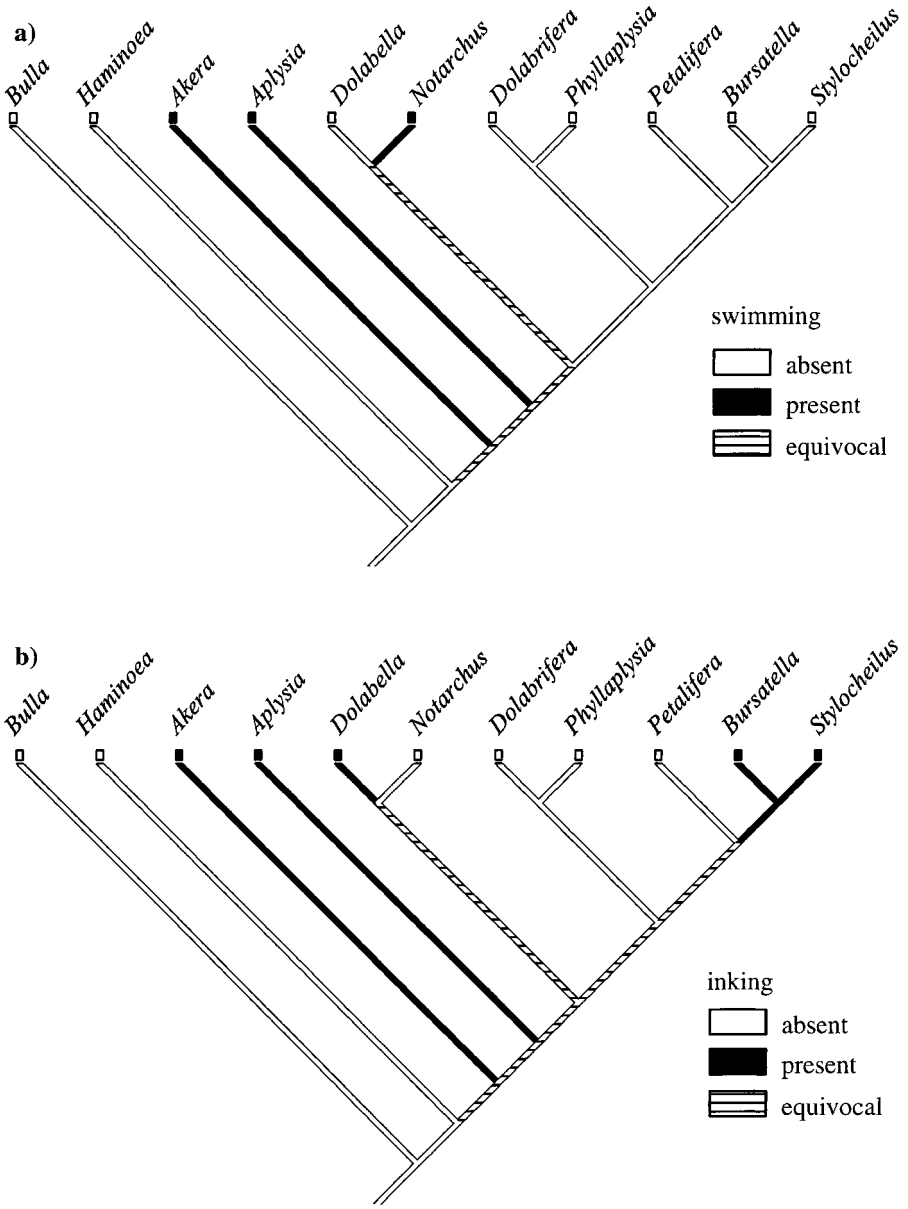


FIG. 8. Mapping of behavioral traits onto the molecular (rRNA) phylogeny. Character tracing was performed in MacClade 3.06. Equivocal cycling was used. (a) Swimming; (b) inking (purple ink).

Relevance of This Study to Neurobiology

Because of the vast amount of information on neurobiology and behavior of sea hares, especially from the genus *Aplysia*, the need for a phylogenetic hypothesis for this group has been pointed out by neurobiologists (Kandel, 1979). Kandel (1979) gave an overview of the available knowledge on different learning mechanisms in *Aplysia* and emphasized the importance of comparative studies in order to understand the evolution of such mechanisms. These types of studies have started to emerge in the literature (Nambu and Scheller, 1986; Wright *et al.*, 1996). The available information on the nervous system of *Aplysia* includes maps of a great number of the giant neurons in this taxa. The behavioral function of many of these neurons has been identified. This knowledge creates ample opportunity for comparative studies because neuronal homology can be determined with high levels of certainty (Wright *et al.*, 1996). For instance, Wright *et al.* (1996) looked at the effects of a neuromodulatory transmitter, serotonin, on spike duration (time span of an action potential) and excitability (number of action potentials for an intracellular depolarizing current) in tail sensory neurons that are involved in defensive withdrawal reflexes. They were able demonstrate that learning-related mechanisms common to most taxa in the anaspidean clade

were lost in *Dolabrifera* by mapping these characters on a partial phylogeny based on morphological characters (Wright *et al.*, 1996). Wright *et al.*'s (1996) pioneering study demonstrates the potential for evolutionary studies of behavioral mechanisms at the neurological level in the well-known sea hare system. The molecular evidence presented in this study provides a phylogenetic hypothesis for the evolution of the anaspidean order which can now be used as a framework for comparative studies of neurological traits in this opisthobranch group.

ACKNOWLEDGMENTS

We are especially grateful to T. Gosliner, J. Ortea, S. Pennings, N. Strenth, K. Lawrence, and T. Capo, who collected some of the specimens used in this study. J. Silberman assisted in various laboratory procedures. We are also thankful to D. Swofford for allowing the use of the beta version of PAUP*. We thank R. Willan for helpful comments on anaspidean taxa and for sharing material in press. We also thank K. Rosen, T. Collins, J. Fell, D. Olson, and two anonymous reviewers for their helpful comments on a previous draft of the manuscript. This paper was prepared by Mónica Medina in partial fulfillment of requirements for the Ph.D. in Marine Biology and Fisheries at the University of Miami. Support for this project was funded by NIH RR 10294.

REFERENCES

Avila, C. (1995). Natural products of opisthobranch molluscs: A biological review. *Annu. Rev. Oceanogr. Mar. Biol.* **33:** 487–559.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., and Saunders, N. C. (1987). Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* **18:** 489–522.

Bebbington, A. (1974). Aplysiid species from East Africa with notes on the Indian Ocean Aplysiomorpha (Gastropoda: Opisthobranchia). *Zool. J. Linn. Soc.* **54:** 63–99.

Bebbington, A. (1977). Aplysiid species from Eastern Australia with notes on the Pacific Ocean Aplysiomorpha (Gastropoda, Opisthobranchia). *Trans. Zool. Soc. London* **34:** 87–147.

Bedford, J. A., and Lutz, P. L. (1992). Respiratory physiology of *Aplysia californica* (J. E. Morton and C. M. Yonge, 1964) and *Aplysia brasiliana* (J. E. Morton and C. M. Yonge, 1964) upon aerial exposure. *J. Exp. Mar. Biol. Ecol.* **155:** 239–248.

Beeman, R. D. (1963). Variation and synonymy of *Phyllaplysia* in the Northeastern Pacific. *Veliger* **3:** 43–47.

Beeman, R. D. (1968). The order Anaspidea. *Veliger* **3** [**Suppl. Pt 2**]: 87–102.

Boss, K. (1982). Mollusca. *In* "Synopsis and Classification of Living Organisms" (S. P. Parker, Ed.), Vol. 1, pp. 945–1161. McGraw–Hill, New York.

Brown, W. M. (1985). The mitochondrial genome of animals. *In* "Molecular Evolutionary Genetics" (R. J. MacIntyre, Ed.), pp. 95–130. Plenum, New York.

Burn, R. (1989). Opisthobranchs (Subclass Opisthobranchia). *In* "Marine Invertebrates of Southern Australia" (S. A. Shepard and I. M. Thomas, Eds.), Part II, pp. 725–788. South Australian Government Printing Division, Adelaide, Australia.

Carefoot, T. (1987). *Aplysia:* Its biology and ecology. *Oceanogr. Mar. Biol. Annu. Rev.* **25:** 167–284.

Carefoot, T. H. (1991). Blood-glucose levels in the Sea Hare *Aplysia dactylomela:* Interrelationships of activity, diet choice and food quality. *J. Exp. Mar. Biol. Ecol.* **154:** 231–244.

Carew, T., and Kandel, E. (1977). Inking in *Aplysia californica.* I. Neural circuit of an all-or-none behavioral response. *J. Neurophysiol.* **40:** 692–707.

Chapman, D. J., and Fox, D. L. (1969). Bile pigment metabolism in the sea-hare *Aplysia. J. Exp. Mar. Biol. Ecol.* **4:** 71–78.

Chastain, M., and Tinoco, I. (1991). Structural elements in RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **41:** 131–177.

Dixon, M. T., and Hillis, D. M. (1993). Ribosomal RNA secondary structure: Compensatory mutations and implications for phylogenetic analysis. *Mol. Biol. Evol.* **10:** 256–267.

Eales, N. (1944). Aplysiids from the Indean Ocean, with a review of the family Aplysiidae. *Proc. Malcol. Soc. London* **26:** 1–22.

Eales, N. B. (1960). Revision of the world species of *Aplysia* (Gastropoda, Opistobranchia). *Bull. Br. Mus. Nat. Hist. Zool.* **5:** 266–404.

Farris, J. S., Kallersjo, M., Kluge, A. G., and Bult, C. (1995). Testing significance of incongruence. *Cladistics* **10:** 315–319.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

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

Gamkrelidze, G., Laurienti, P. J., and Blankenship, J. E. (1995). Identification and characterization of cerebral ganglion neurons that induce swimming and modulate swim-related pedal ganglion neurons in *Aplysia brasiliana. J. Neurophysiol.* **74:** 1444–1462.

Geiger, D. L., and Jung, P. (1996). A shell of *Floribella aldrichi* (Dall, 1890), a large sea hare (mollusca: opisthobranchia: aplysiidae) from the neogene of the northern Dominican Republic. *J. Conch. Lond.* **35:** 437–444.

Ghiselin, M. T. (1965). Reproductive function and the phylogeny of opisthobranch gastropods. *Malacologia* **3:** 327–378.

Gosliner, T. M. (1981). Origins and relationships of primitive members of the opisthobranchia (Mollusca: Gastropoda). *Biol. J. Linn. Soc.* **16:** 197–225.

Gosliner, T. M. (1985). Parallelism, parsimony and the testing of phylogenetic hypothesis: The case of opistobranch gastropods. *In* "Species and Speciation" (E. S. Vrba, Ed.), pp. 105–107. Museum Monograph No. 4, Transvall Museum, Pretoria.

Gosliner, T. M. (1991). Morphological parallelism in opistobranch gastropods. *Malacologia* **32:** 313–327.

Gutell, R. R. (1994). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Res.* **22:** 3502–3507.

Hamilton, P. V., and Ambrose, H. W. (1975). Swimming and orientation in *Aplysia brasiliana* (Mollusca: Gastropoda). *Mar. Behav. Physiol.* **3:** 131–144.

Hennig, W. (1966). "Phylogenetic Systematics," Univ. of Illinois Press, Urbana.

Hickson, R. E., Simon, C., Cooper, A., Spicer, G. S., Sullivan, J., and Penny, D. (1996). Conserved sequence motifs, alignment, and secondary structure for the third domain of animal 12S rRNA. *Mol. Biol. Evol.* **13:** 150–169.

Higgins, D. G., and Sharp, P. M. (1988). CLUSTAL: A package for performing multiple sequence alignments on a microcomputer. *Gene* **73:** 237–244.

Hillis, D. M., and Dixon, M. T. (1991). Ribosomal DNA: Molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66:** 411–453.

Kandel, E. (1979). "Behavioral Biology of *Aplysia*," Freeman, New York.

Kjer, K. M. (1995). Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: An example of alignment

and data presentation from the frogs. *Mol. Phylogenet. Evol.* **4:** 314–330.

Kluge, A. G., and Farris, J. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst. Zool.* **18:** 1–32.

Kumar, S., Tamura, K., and Nei, M. (1993). MEGA: Molecular Evolutionary Genetics Analysis. The Pennsylvania State Univ., University Park, PA.

Marcus, E. D. B. R. (1972). On the Anaspidea (Gastropoda: Opisthobranchia) of the warm waters of the Western Atlantic. *Bull. Mar. Sci.* **22:** 841–874.

Martin, R. (1966). On the swimming behavior and biology of *Notharcus punctatus* Philippi (Gastropoda, Opisthobranchia). *Pubbl. Satz. Zool. Napoli* **35:** 61–75.

Mindell, D. P., and Honeycutt, R. L. (1990). Ribosomal RNA in vertebrates: Evolution and phylogenetic applications. *Annu. Rev. Ecol. Syst.* **21:** 541–566.

Mikkelsen, P. M. (1993). Monophyly versus the Cephalaspidea (Gastropoda, Opistobranchia) with an analysis of traditional Cephalaspid characters. *Boll. Malacol.* **29:** 115–138.

Mikkelsen, P. M. (1996). The evolutionary relationships of the Cephalaspidea S.L. (Gastropoda: Opisthobranchia): A phylogenetic analysis. *Malacologia* **37:** 375–442.

Moritz, C., T. E., D., and W. M., B. (1987). Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. *Annu. Rev. Ecol. Syst.* **18:** 269–292.

Morton, J. E., and Holme, N. A. (1955). The occurrence at Plymouth of the opisthobranch *Akera bullata* with notes on its habits and relationships. *J. Mar. Biol. Assoc. U.K.* **34:** 101–112.

Morton, J. E. (1972). The form and functioning of the pallial organs in the opisthobranch *Akera bullata* with a discussion in the nature of the gill in Notaspidea and other techtibranchs. *Veliger* **14:** 337–349.

Nambu, J. R., and Scheller, R. H. (1986). Egg-laying hormone genes of *Aplysia:* Evolution of the ELH gene family. *J. Neurosci.* **6:** 2026–2036.

Nolen, T. G., Johnson, P. M., Kicklighter, C. E., and Capo, T. (1995). Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. *J. Comp. Physiol. A* **176:** 239–254.

Ortí, G., Petry, P., Port, J. I. R., Jegu, M., and Meyer, A. (1996). Patterns of nucleotide change in mitochondrial ribosomal RNA genes and the phylogeny of piranhas. *J. Mol. Evol.* **42:** 169–182.

Ortí, G., and Meyer, A. (1997). The radiation of characiform fishes and the limits of resolution of mitochondrial ribosomal DNA sequences. *Syst. Biol.* **46:** 75–100.

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., and Grabowski, G. (1991). "The Simple Fool's Guide to PCR," Version 2.0. Department of Zoology and Kewalo Marine Laboratory, Univ. of Hawaii.

Pennings, S. C. (1994). Interspecific variation in chemical defenses in the sea hares (Opisthobranchia: anaspidea). *J. Exp. Mar. Biol. Ecol.* **180:** 203–219.

Pennings, S. C., and Paul, V. J. (1992). Effect of plant toughness, calcification, and chemistry on herbivory by *Dolabella auricularia*. *Ecology* **73:** 1606–1619.

Pennings, S. C., and Paul, V. J. (1993). Secondary chemistry does not limit dietary range of the specialist sea hare *Stylocheilus longicauda* (Quoy et Gaimard 1824). *J. Exp. Mar. Biol. Ecol.* **174:** 97–113.

Provut-Fol, A. (1934). Les opisthobranches de Quoy et Gaimard. *Arch. Mus. Hist. Nat.* **11:** 3–92.

Pruvot-Fol, A. (1954). "Mollusques Opisthobranches. Faune de France," Vol. 58, Paul Lechevalier, Paris.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schmekel, L. (1985). Aspects of evolution within the opisthobranchs. *In* "The Mollusca" Vol. 10, "Evolution" (E. R. Trueman and M. R. Clarke, Eds.), Academic Press, Orlando.

Seutin, G., White, B. N., and Boag, P. T. (1991). Preservation of avian blood and tissue samples for DNA analysis. *Can. J. Zool.* **69:** 82–90.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., and Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* **87:** 6651–701.

Sorenson, M. D. (1996). TreeRot. Univ. of Michigan, Ann Arbor.

Strimmer, K., and von Haeseler, A. (1996). Quartet puzzling: A quartet maximum-likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13:** 964–969.

Strimmer, K., and von Haeseler, A. (1997). Puzzle. Maximum likelihood analysis for nucleotide and amino acid alignments. Zoologisches Institut, Munchen, Germany.

Swofford, D. L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony. Version 4.0d64. Sinauer, Sunderland, MA.

Thompson, T. E. (1976). "Biology of Opisthobranch Molluscs," Vol. 1, Ray Society, London.

Tracey, S., Todd, J. A., and Erwin, D. H. (1993). Mollusca: Gastropoda. *In* "The Fossil Record 2" (M. J. Benton, Ed.), pp. 131–167. Chapman & Hall, London.

Van de Peer, Y., Van den Broeck, I., De Rijk, P., and De Wachter, R. (1994). Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res.* **22:** 3488–3494.

Vawter, L., and Brown, W. M. (1993). Rates and patterns of base change in the small subunit ribosomal RNA gene. *Genetics* **134:** 597–608.

Von der Porten, K., and Parsons, D. W. (1982). Swimming in *Aplysia brasiliana:* Analysis of behavior and neuronal pathways. *Behav. Neural Biol.* **36:** 1–23.

Wenz, W. (1938). "Gastropoda," Borntraeger, Berlin.

Wheeler, W. C., and Honeycutt, R. L. (1988). Paired sequence difference in ribosomal RNAs: Evolutionary and phylogenetic implications. *Mol. Biol. Evol.* **5:** 90–96.

Willan, R. C. (1998). Order Anaspidea. *In* "Fauna of Australia." Mollusca, vol. 5, pp. 62–64. Australian Publishing Service, Canberra.

Williams, G. C. (1975). "Phylogenetic Implications of the Degree of Concentration within the Opisthobranch Nervous System," Master of Arts thesis, San Francisco State Univ.

Wright, W. G., Kirschman, D., Rozen, D., and Maynard, B. (1996). Phylogenetic analysis of learning-related neuromodulation in molluscan mechanosensory neurons. *Evolution* **50:** 2248–2263.