Molecular Phylogeny of Euthyneura (Mollusca: Gastropoda)

Cristina Grande, José Templado,* J. Lucas Cervera,† and Rafael Zardoya**

*Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain;
†Departamento de Biología, Facultad de Ciencias del Mar, Universidad de Cádiz, Cádiz, Spain

A new phylogenetic hypothesis for Euthyneura is proposed based on the analysis of primary sequence data (mitochondrial *cox1, trnV, rrnL, trnL(cun), trnA, trnP, nad6*, and *nad5* genes) and the phylogenetic utility of two rare genomic changes (the relative position of the mitochondrial *trnP* gene, and an insertion/deletion event in a conserved region of the mitochondrial Cox1 protein) is addressed. Both sources of phylogenetic information clearly rejected the monophyly of pulmonates, a group of gastropods well supported so far by morphological evidence. The marine basommatophoran pulmonate *Siphonaria* was placed within opisthobranchs and shared with them the insertion of a Glycine in the Cox 1 protein. The marine systellommatophoran pulmonate *Onchidella* was recovered at the base of the opisthobranch + *Siphonaria* clade. Opisthobranchs, *Siphonaria*, and *Onchidella* shared the relative position of the mitochondrial *trnP* gene between the mitochondrial *trnA* and *nad6* genes. The land snails and slugs (stylommatophoran pulmonates) were recovered as an early split in the phylogeny of advanced gastropods. The monophyly of the Euthyneura (Opisthobranchia + Pulmonata) was rejected by the inclusion of the heterostrophan *Pyramidella*.

Introduction

Molecular data have proved to be very helpful in resolving phylogenetic relationships among different taxa at multiple hierarchical levels. Most molecular phylogenetic hypotheses are based on the comparative analysis of the pattern of nucleotide or amino acid substitutions along sequences. However, phylogenetic inferences based on primary sequence data are known to have at least two specific limitations: (1) saturation caused by multiple-hits, which renders homoplasious changes, and (2) uncertainty in positional homology assignment at indel-rich regions, usually with high rates of substitution. The potential negative effect of these two problems becomes more relevant at progressively higher taxonomic levels (Boore and Brown 1998).

In cases where phylogenetic analyses based on sequence substitutions render conflicting results, rare genomic changes (RGCs) may be very helpful in discerning among alternative phylogenetic hypotheses (see Rokas and Holland [2000] for a review). Insertion/deletion (indel) events within well-conserved regions of the genome and gene rearrangements are two examples of RGCs that have been successfully used to resolve deep phylogenetic relationships (Rivera and Lake 1992; Macey et al. 1997; Boore and Brown 1998; Keeling and Palmer 2000; Venkatesh, Erdmann, and Brenner 2001).

The potential of RGCs for phylogenetic reconstruction has been tested in gastropods, a group of mollusks known to have high sequence substitution rates (Thomaz, Guiller, and Clarke 1996; Davison 2002). For instance, the absence or reduction of entire stem/loop structures in several domains of the secondary structure of the mitochondrial large subunit rRNA has been shown to be a molecular synapomorphy of derived gastropods (Lydeard et al. 2000, 2002*b*). Moreover, mitochondrial DNA gene rearrangements have been useful in identifying phylogenetic affinities among several gastropod groups at different taxonomic levels (Kurabayashi and Ueshima 2000*b*; Rawlings, Collins, and Bieler 2001; Lydeard et al. 2002*a*).

Gastropods were traditionally classified into three main subclasses, Prosobranchia, Pulmonata, and Opisthobranchia (Thiele 1931). However, recent morphological studies rejected the monophyly of Prosobranchia (Haszprunar 1988) and failed to recover the monophyly of Opisthobranchia (Salvini-Plawen and Steiner 1996; Ponder and Lindberg 1997; Dayrat and Tillier 2002; fig. 1*A*). According to new morphological data, the monophyletic Pulmonata (Salvini-Plawen 1970; Tillier 1984; Haszprunar 1985; Haszprunar and Huber 1990; Nordsieck 1992; Dayrat and Tillier 2002) and the paraphyletic Opisthobranchia (Boettger 1955; Haszprunar 1988; Salvini-Plawen and Steiner 1996; Ponder and Lindberg 1997) are clearly distinct from the remaining gastropods and are grouped together in the clade Euthyneura Spengel 1881. The monophyly of Euthyneura is generally accepted and is supported by several morphological synapomorphies (Gosliner 1981; Haszprunar 1988; Salvini-Plawen and Steiner 1996; but see Dayrat and Tillier 2002). Euthyneura together with the paraphyletic group Heterostropha (pyramidelids and other related groups) define the clade Heterobranchia (Haszprunar 1985).

Opisthobranchs are almost exclusively marine organisms, with the only exception being few freshwater acochlidians (Rudman and Willan 1998). The reduction or loss of the shell, an extensive body reorganization, and the acquisition of chemical defenses are evolutionary trends shared to different degrees by most opisthobranch clades. In contrast, pulmonates are mainly land and freshwater gastropods, although there are a few marine groups. All pulmonates share the presence of a lung as the respiratory surface. Most of the species have a shell, although it is modified or absent in some groups.

Most phylogenetic analyses based on nuclear 18S and 28S rRNA gene sequences rejected the monophyly of both Opisthobranchia and Pulmonata (Tillier and Ponder 1992; Rosenberg et al. 1994; Tillier et al. 1994; Tillier, Masselot, and Tillier 1996; Winnepenninckx et al. 1998; Wollscheid and Wägele 1999; Yoon and Kim 2000; Dayrat et al. 2001;

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E-mail: cristina@mncn.csic.es.

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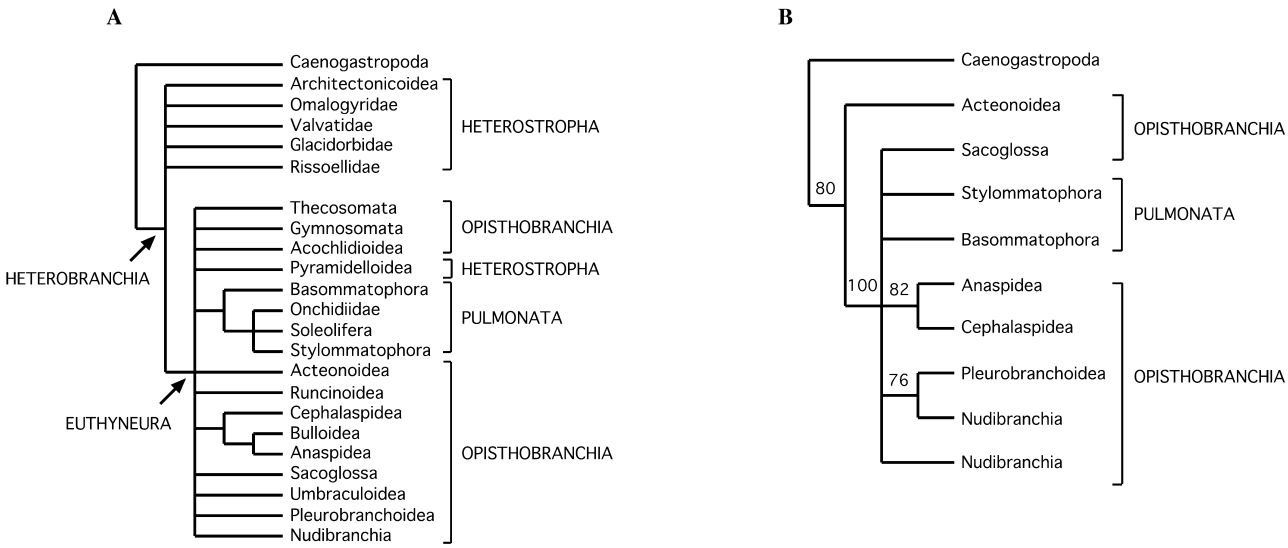

FIG. 1.—Phylogenetic relationships among gastropods based on (*A*) morphological data (Dayrat et al. 2002) and (*B*) molecular data (Thollesson 1999). For the molecular phylogeny, nodes with bootstrap values below 50% were forced to collapse.

Wollscheid et al. 2001). However, these analyses rendered conflicting results, and their conclusions lacked sufficient support because of the low resolution of nuclear rRNA genes at this taxonomic level. In contrast, a recent study (Wade and Mordan 2000) based on a nuclear fragment including partial 5.8S rDNA, complete ITS-2, and partial large subunit rDNA sequences supported the monophyly of the above-mentioned gastropod groups. Thollesson (1999) proposed a phylogenetic hypothesis for Euthyneura based on a small fragment (480 bp) of the mitochondrial *rrnL* gene and rejected the monophyly of opisthobranchs (fig. 1*B*). In contrast, Grande et al. (2002) tentatively concluded that opisthobranchs are monophyletic based on the phylogenetic analyses of the mitochondrial *cox1*, *rrnL*, *nad6*, and *nad5* genes from several species representing five different orders of opisthobranchs. These conflicting results may be clarified and resolved if more representatives of each gastropod group are included in the phylogenetic analyses, if large sequence data sets are compiled, and if the potential phylogenetic utility of RGCs is explored, as suggested by Grande et al. (2002).

To further understand phylogenetic relationships within derived gastropods, we have determined partial sequences of mitochondrial *cox1* and *nad5* genes and the complete sequences of mitochondrial *trnV*, *rrnL*, *trnL(cun)*, *trnA*, *trnP*, and *nad6* genes for representative species of the main orders of Pulmonata and Opisthobranchia, as well as the heterostrophan *Pyramidella dolabrata*. The new sequences were analyzed with current methods of phylogenetic inference and screened for RGCs.

Materials and Methods

Samples and DNA Extraction

A total of 42 species of derived gastropods were sequenced anew in this study (table 1). Sequence data from the complete mitochondrial genomes of the opisthobranchs *Pupa strigosa* (Kurabayashi and Ueshima 2000*a*), and *Roboastra europaea* (Grande et al. 2002); the pulmonates *Cepaea nemoralis* (Terrett, Miles, and Thomas 1996), and *Albinaria coerulea* (Hatzoglou, Rodakis, and Lecanidou 1995); the squid *Loligo bleekeri* (Sasuga et al. 1999), and the partial mitochondrial genome of the caenogastropodan *Littorina saxatilis* (Wilding, Mill, and Grahame 1999) were also included in the phylogenetic analyses (table 1). For each specimen, tissue was grounded in liquid nitrogen, and resuspended in 500 μl of extraction buffer (Towner 1991). After homogenization, total cellular DNA was purified by a standard phenol/chloroform procedure followed by an ethanol precipitation.

Polymerase Chain Reaction Amplification, Cloning, and Sequencing

Five overlapping DNA fragments (fig. 2) were amplified by polymerase chain reaction (PCR) with the following sets of primers: primers LCO-1490 and HCO-2198 (Folmer et al. 1994); OPISA-F, 5′-GGGGCAATTAATTTTATTAC-3′ and OPISA-R, 5′-ACCATTATACAAAAGGT-3′; OPIS COI-F and OPIS1-R (Grande et al. 2002); 16Sar-L and 16Sbr-H (Palumbi et al. 1991); LP-F (Grande et al. 2002) and OPISB-R, 5′-ACTCCTAGCCCATCTCANCC-3′.

Standard PCR reactions containing 67 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 2.5 μM of each primer, template DNA (10–100 ng), and Taq DNA polymerase (1 unit, Biotools) in a final volume of 25 μl were subjected to 40 cycles of denaturing at 94°C for 60 s, annealing at 42°–52°C for 60 s, and extending at 72°C for 90 s. The PCR products were ethanol precipitated and, either directly sequenced using the corresponding PCR primers or cloned into the pGEM-T vector (Promega) and sequenced using the M13 universal (forward and reverse) sequencing primers. DNA sequences of both strands were obtained using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems, Inc.) on an automated DNA sequencer (Applied Biosystems Prism 3700) according to the manufacturer's instructions.

Table 1
List of Samples Analyzed in This Study

Species	Locality	Sequenced PCR Fragment*
Opisthobranchia		
Architectibranchia		
Pupa strigosa	—	AB028237
Micromelo undata	Cape Verde Islands	1–4
Cephalaspidea		
Haminoea callidegenita	Pontevedra, N Spain	1–4
Philine aperta	Murcia, SE Spain	1–4
Chelidonura africana	Porto Santo, Madeira Islands	1, 2, AY098930
Runcina coronata	Sagres, Portugal	1–4
Anaspidea		
Aplysia punctata	Pontevedra, NW Spain	1, 2, AY098931
Petalifera petalifera	Murcia, SE Spain	1–4
Dolabrifera dolabrifera	Cape Verde Islands	1–4
Sacoglossa		
Ascobulla fragilis	Murcia, SE Spain	1, 2, AY098929
Tylodinoidea		
Umbraculum mediterraneum	Gerona, NE Spain	1, 2, AY098928
Tylodina perversa	Porto Santo, Madeira Islands	1–4
Pleurobranchoidea		
Berthella plumula	Pontevedra, NW Spain	1–5
Pleurobranchaea meckeli	Gerona, NE Spain	1–4
Bathyberthella antartica	Antarctica	1–4
Nudibranchia		
Aeolidia papillosa	Pontevedra, NW Spain	1, 2, AY098927
Roboastra europaea	—	AY083457
Ancula gibbosa	Kingsbarns, Scotland	1–4
Doris pseudoargus	Kingsbarns, Scotland	1–4
Facelina bostoniensis	Clachan Seil, Scotland	1–4
Tergipes tergipes	Clachan Seil, Scotland	1–4
Onchidoris muricata	Clachan Seil, Scotland	1–4
Cadlina laevis	Kinkell Braes, Scotland	1–4
Tethys fimbria	Tarragona, NE Spain	1–4
Chromodoris krohni	Murcia, SE Spain	1–4
Flabellina affinis	Murcia, SE Spain	1–4
Platydoris argo	Ceuta, S Spain	1–4
Tambja ceutae	Porto Santo, Madeira Islands	1–4
Aldisa banyulensis	Porto Santo, Madeira Islands	1–4
Discodoris confusa	Porto Santo, Madeira Islands	1–4
Dendronotus frondosus	Oban, Scotland	1–4
Favorinus branchialis	Oban, Scotland	1–4
Cuthona ocellata	Sagres, Portugal	1–4
Rostanga pulchra	California, USA	1–4
Triopha maculata	California, USA	1–4
Eubranchus sp.	Sagres, Portugal	1–4
Hancockia uncinata	Sines, Portugal	1–4
Pulmonata		
Systelommatophora		
Onchidella celtica	Ceuta, S Spain	1–5
Basommatophora		
Siphonaria pectinata	Ceuta, S Spain	1–5
Eupulmonata		
Rumina decollata	Assumar, Portugal	1–4
Elona quimperiana	Asturias, N Spain	1–4
Helix aspersa	Pontevedra, NW Spain	1–5
Myosotella myosotis	Pontevedra, NW Spain	1–4
Cepaea nemoralis	—	NC_001816
Albinaria coerulea	—	NC_001761
Other Gastropoda		
Heterostropha		
Pyramidella dolabrata	Annobon Island, Gulf of Equatorial Guinea	1–5
Caenogastropoda		
Littorina saxatilis	—	AJ132137
Other Mollusca		
Loligo bleekeri	—	NC_002507

* Numbers correspond to fragments in figure 2 or to accession entries from GenBank.

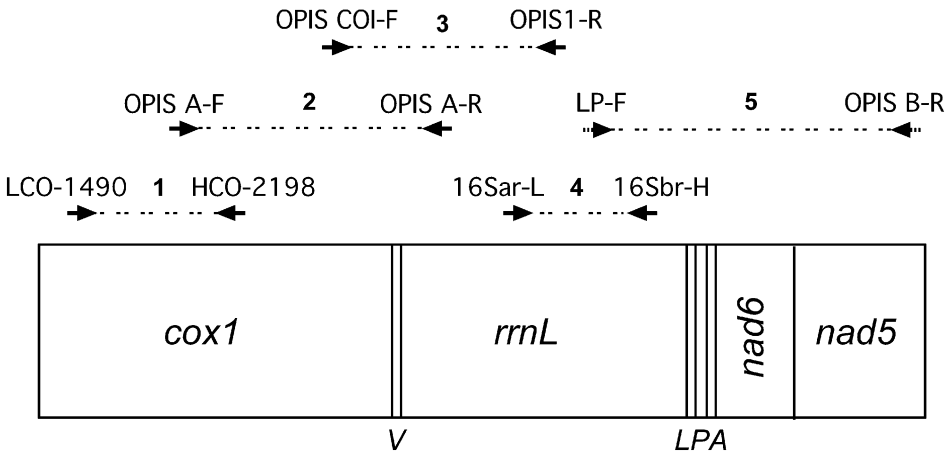

FIG. 2.—Mitochondrial DNA fragment amplified in this work and sequencing strategy. Arrows denote the localization and direction of the primers used in the PCR amplification. See text for the primer sources.

Molecular and Phylogenetic Analyses

Gene boundaries were determined by comparison with other gastropod mitochondrial genomes. Protein-coding genes were delimited by their start and stop codons. Deduced tRNAs were folded into their corresponding cloverleaf secondary structure.

Nucleotide (for the mitochondrial *rrnL* gene) and deduced amino-acid sequences (for the mitochondrial *cox1, nad6*, and *nad5* genes) were aligned using ClustalX version 1.62b (Thompson et al. 1997) followed by refinement by eye. Ambiguous alignments and gaps were excluded from the analysis using Gblocks 0.73b (Castresana 2000). Modeltest version 3.06 (Posada and Crandall 1998) was used to estimate the evolutionary model that best fit the nucleotide (mitochondrial *rrnL* gene) data set. The Akaike information criteria (AIC) implemented in Modeltest selected the GTR + I + Γ (Rodriguez et al. 1990) evolutionary model.

Bayesian inferences (BI) of gastropod phylogeny were performed with MrBayes 3.0b3 (Huelsenbeck and Ronquist 2001) by Metropolis coupled Markov chain Monte Carlo (MCMCMC) sampling for 1 million generations (four simultaneous MC chains; sample frequency 100; burn-in 1,000 generations). Bayesian analyses were run independently at least twice, beginning with different starting trees (Huelsenbeck and Bollback 2001). We used GTR + I + Γ (for the *rrnL* nucleotide sequence data) and mtREV (Adachi and Hasegawa 1996) (for the *cox1, nad6*, and *nad5* amino-acid sequence data) as evolutionary models. Support for tree nodes was determined based on the values of Bayesian posterior probability (BPP) obtained from a majority-rule consensus tree with MrBayes 3.0b3.

To further confirm the gastropod trees reconstructed by the Bayesian method of inference, the deduced amino-acid sequences of mitochondrial protein-coding genes (*cox1, nad6*, and *nad5*) were combined into a single data set that was subjected to maximum-parsimony (MP) and minimum evolution (ME) methods of phylogenetic inference. The MP analyses were performed with PAUP* 4.0b10 (Swofford 2002) using heuristic searches (TBR branch swapping; MulTrees option in effect) with 10 random additions of taxa. The ME analyses (Rzhetsky and Nei 1992) were carried out with PAUP* 4.0b10 using mean character distances. Robustness of MP and ME analyses was tested by bootstrapping with 1,000 pseudoreplicates each (Felsenstein 1985).

The sequences reported in this article have been deposited in the GenBank database (accession nos. AY345014–AY345055).

Results

A Molecular Phylogeny of Euthyneura

Phylogenetic hypotheses of gastropod relationships were based on two different sequence data sets. The first data set included the deduced amino acid sequences of mitochondrial *cox1* (partial), *nad6* (complete), and *nad5* (partial) genes combined with the nucleotide sequence of the complete mitochondrial *rrnL* gene for 14 gastropod taxa (fig. 3). The squid *Loligo bleekeri* (Sasuga et al. 1999) was used as outgroup because most authors currently consider cephalopods to be the sister group of gastropods (Haszprunar 1988; Bieler 1992). This data set comprised 2,301 positions, of which 1,179 (mostly in the *rrnL* gene) were excluded because of ambiguity in positional homology assignment at gap-rich regions. The second data set included the deduced amino acid sequence of the mitochondrial *cox1* gene (partial), combined with the nucleotide sequence of the *rrnL* gene (complete) for 47 gastropod taxa. This data set comprised 1,986 positions and was reduced to 857 homologous positions after the exclusion of gap-rich regions (mostly in the *rrnL* gene).

The majority-rule consensus tree resulting from the Bayesian inference based on the 15-taxon sequence data set under the GTR + I + Γ (*rrnL* gene) and mtREV (*cox1*, *nad6*, and *nad5* genes) substitution models is presented in figure 3. The recovered topology rejected the monophyly of Euthyneura, Pulmonata, and Opisthobranchia (fig. 3). The relative position of the heterostrophan *Pyramidella dolabrata* deep within Euthyneura (supported by a 100% BPP) made this group paraphyletic (fig. 3). Pulmonates

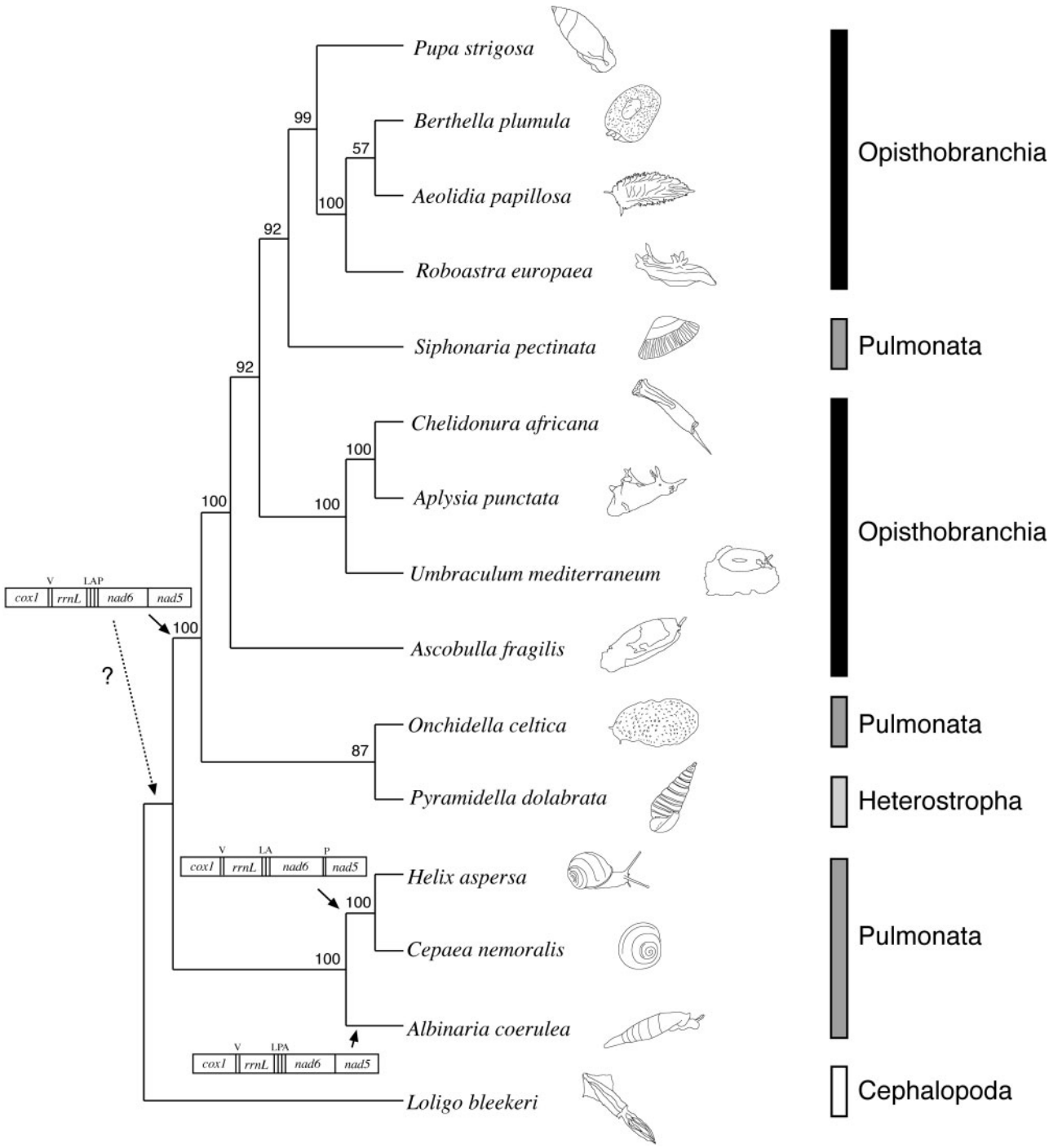

FIG. 3.— 50% majority rule Bayesian tree for 14 species of gastropods inferred from a combined data set including sequences of the mitochondrial *rrnL* gene (GTR + I + Γ) and the deduced amino-acid sequences of mitochondrial *cox1*, *nad6*, and *nad5* genes (mt-REV). The numbers above branches represent Bayesian posterior probabilities (only values above 95% are statistically significant). The squid, *Loligo bleekeri* was used as outgroup. Mitochondrial tRNA gene rearrangements were mapped onto the inferred phylogeny. A question mark indicates the possibility that the *rrnL*, *trnL*, *trnA*, *trnP*, *nad6*, *nad5* gene order may be the ancestral state for Heterobranchia.

were recovered as a polyphyletic group. The inclusion of the marine basommatophoran pulmonate *Siphonaria pectinata* within the Opisthobranchia (100% BPP) made this group paraphyletic (fig. 3). The marine systelommatophoran pulmonate *Onchidella celtica* was placed with *P. dolabrata* as sister group of Opisthobranchia + *S. pectinata* (100% BPP) (fig. 3). Land stylommatophoran pulmonates (*Helix* + *Cepaea* + *Albinaria*) were monophyletic (100% BPP) and resolved as the sister group of all other gastropod taxa included in the analysis (fig. 3). A Bayesian analysis under the GTR + I + Γ (*rrnL* gene) and mtREV (*cox1* and *nad6* genes) substitution models, using the same ingroup taxa but with *Littorina* as outgroup, also recovered the Opisthobranchia + *S. pectinata* clade (99%

BPP) as well as the basal position of *O. celtica* and *P. dolabrata* (99% BPP) with respect to Opisthobranchia + *S. pectinata* (not shown).

Both the MP and the ME phylogenetic inferences based on a 15-taxon data set that combined the deduced amino acid sequences of mitochondrial *cox1, nad6*, and *nad5* genes and with *Loligo* as outgroup recovered congruent trees that also supported the Opisthobranchia + *S. pectinata* clade (97% and 100% bootstrap values, respectively) as well as the basal position of *O. celtica* and *P. dolabrata* (83% and 98% bootstrap values, respectively) with respect to Opisthobranchia + *S. pectinata*. The same two nodes were also supported with high bootstrap values when the deduced amino acid sequences of mitochondrial *cox1* and *nad6* genes and the same ingroup taxa were analyzed, with *Littorina* as outgroup (not shown).

The new gastropod phylogeny was further confirmed by a Bayesian inference based on an extended 47-taxon sequence data set. The majority-rule consensus tree resulting from the Bayesian inference under the GTR + I + Γ (*rrnL* gene) and mtREV (*cox1* gene) substitution models is presented in figure 4. The recovered phylogeny was in agreement with that based on the 15-taxon data set. Euthyneura are not monophyletic because of the relative position of the heterostrophan *P. dolabrata*. Pulmonata is polyphyletic with basommatophoran, systelommatophoran, and stylommatophoran lineages recovered in different positions of the tree. The basommatophoran *S. pectinata* was included within the Opisthobranchia with high statistical support (100% BPP). The systelommatophoran pulmonate *O. celtica* together with *P. dolabrata* were placed as the sister group of the Opisthobranchia + *S. pectinata* clade (90% BPP). Stylommatophoran pulmonates are monophyletic (100% BPP), and together with the marine ellobioideid pulmonate *Myosotella myosotis*, were placed as the most basal of the analyzed gastropod lineages. Within Opisthobranchia, the order Sacoglossa was recovered as the most basal group (fig. 4). The orders Anaspidea, Tylodinoidea, and Cephalaspidea formed a well-supported clade (100% BPP). The validity of the order Architectibranchia as an independent opisthobranch lineage was confirmed by our analysis. The order Pleurobranchoidea was located within Nudibranchia, rendering the latter paraphyletic (opisthobranch intrarelationships will be further analyzed elsewhere).

trnP Gene Rearrangements

A fragment of 3,600 bp including partial sequences of the mitochondrial *cox1* and *nad5* genes and the complete sequences of the mitochondrial *trnV*, *rrnL*, *trnL(cun)*, *trnA*, *trnP*, and *nad6* genes was analyzed for 14 species of derived gastropods. The most striking result from the comparative analysis of these sequences was the variable position of the mitochondrial *trnP* gene in the different gastropod lineages that defined three distinct groups (fig. 3). All analyzed opisthobranchs, the pulmonates *Siphonaria pectinata* (order Basommatophora) and *Onchidella celtica* (order Systelommatophora), as well as the heterostrophan *Pyramidella dolabrata* shared the same gene order with the *trnP* gene between the *trnA* and *nad6* genes (fig. 3). The stylommatophoran pulmonates *Cepaea nemoralis* and *Helix aspersa* shared the same gene order with the *trnP* gene located between the *nad6* and *nad5* genes (fig. 3). Finally, the stylommatophoran pulmonate *Albinaria coerulea* presented a unique gene order with the *trnP* gene between *trnL(cun)* and *trnA* genes (fig. 3).

An Indel in the Deduced Amino Acid Sequence of the *cox1* Gene

The deduced amino acid sequences of the *cox1*, *nad6*, and *nad5* genes of the same 14 species of derived gastropods were screened for indels. A single informative amino-acid indel was found at position 46 of the mitochondrial Cox1 protein (numbered according to the *Roboastra europaea* Cox1 amino acid sequence). The indel in the Cox1 protein was further analyzed in a larger data set that included 47 gastropod species. All analyzed opisthobranchs, as well as the pulmonate *Siphonaria pectinata*, had a Glycine at that position (fig. 5). The remaining pulmonates and the heterostrophan *Pyramidella dolabrata* had a deletion (fig. 5). The caenogastropodan *Littorina saxatilis* had a Glycine at that position, and the squid *Loligo bleekeri* had an Asparragine at that position. We searched GenBank for additional gastropod *cox1* gene sequences and found that Caenogastropoda and related groups (formerly included in Prosobranchia), as well as the heterostrophan *Cornirostra pellucida*, shared a Glycine at position 46 of the mitochondrial Cox1 protein. In contrast, all mitochondrial Cox1 amino acid sequences of Pulmonata found in GenBank (except *Siphonaria zelandica*) had a deletion at the above-mentioned position (fig. 5).

Discussion

Here we present a molecular phylogeny of Euthyneura based on mitochondrial sequence data that resolves several controversies regarding phylogenetic relationships of the major lineages of gastropods and supports a revision of

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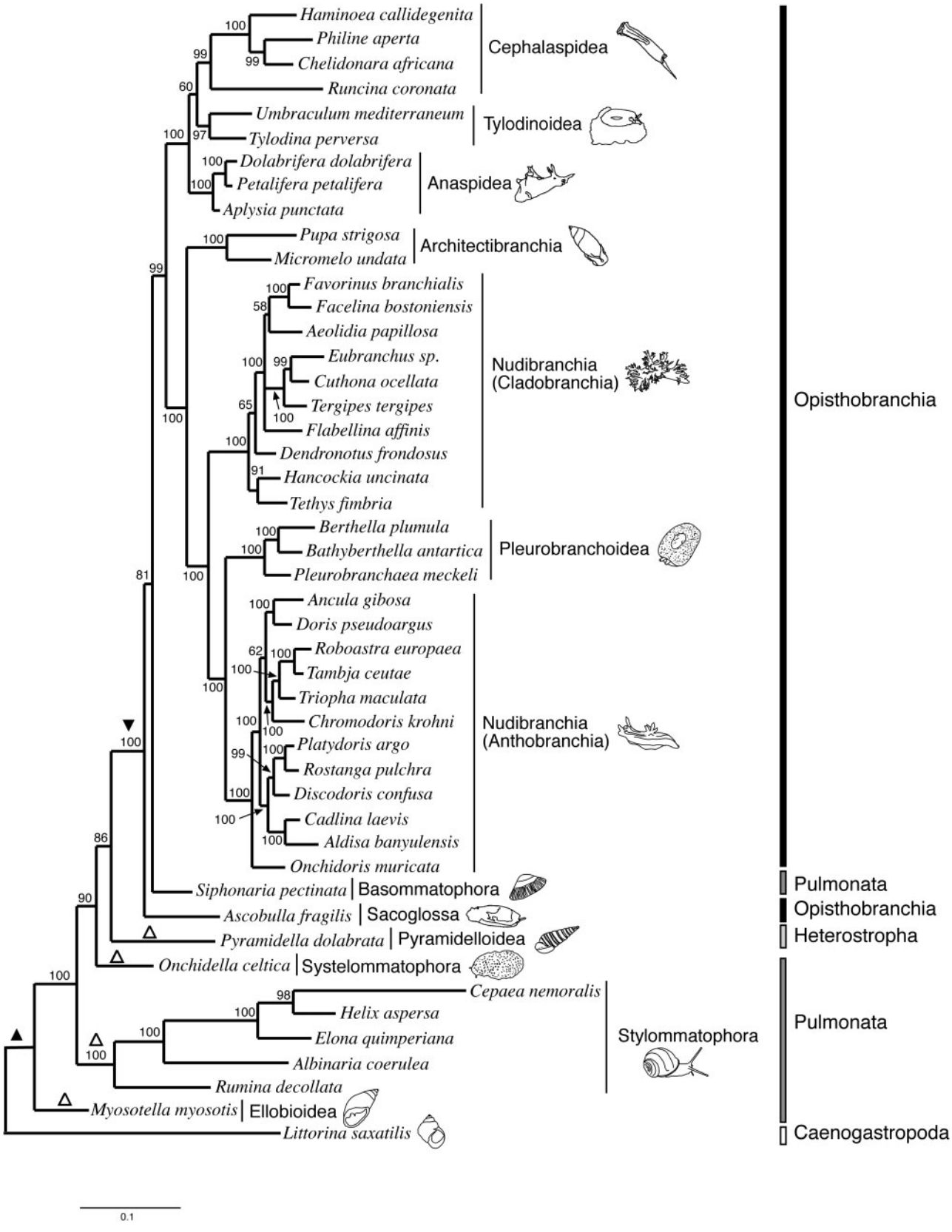
FIG. 4.—Bayesian tree for 47 species of gastropods inferred from the nucleotide sequences of the mitochondrial rrnL gene (GTR + I + Γ) and the deduced amino-acid sequences of the mitochondrial cox1 gene (mt-Rev). The numbers above branches represent Bayesian posterior probabilities (only values above 95% are statistically significant). The Caenogastropod *Littorina saxatilis* was used as outgroup. Filled and open triangles indicate alternative hypotheses for the evolution of the indel at position 46 of the mitochondrial Cox1 protein (numbering corresponds to the amino acid Cox1 sequence of *R. europaea*) (see text). ▲, △ indicate a proposal amino acid deletion. ▼ indicates a proposal insertion.


Haminoea callidegenita
Philine aperta
Chelidonara africana
Runcina coronata
Cephalaspidea
Umbraculum mediterraneum
Tylodina perversa
Tylodinoidea
Dolabrifera dolabrifera
Petalifera petalifera
Aplysia punctata
Anaspidea
Pupa strigosa
Micromelo undata
Architectibranchia
Favorinus branchialis
Facelina bostoniensis
Aeolidia papillosa
Eubranchus sp.
Cuthona ocellata
Tergipes tergipes
Flabellina affinis
Dendronotus frondosus
Hancockia uncinata
Tethys fimbria
Nudibranchia (Cladobranchia)
Berthella plumula
Bathyberthella antartica
Pleurobranchaea meckeli
Pleurobranchoidea
Ancula gibosa
Doris pseudoargus
Roboastra europaea
Tambja ceutae
Triopha maculata
Chromodoris krohni
Platydoris argo
Rostanga pulchra
Discodoris confusa
Cadlina laevis
Aldisa banyulensis
Onchidoris muricata
Nudibranchia (Anthobranchia)
Siphonaria pectinata | Basommatophora
Ascobulla fragilis | Sacoglossa
Pyramidella dolabrata | Pyramidelloidea
Onchidella celtica | Systelommatophora
Cepaea nemoralis
Helix aspersa
Elona quimperiana
Albinaria coerulea
Rumina decollata
Stylommatophora
Myosotella myosotis | Ellobioidea
Littorina saxatilis
Opisthobranchia
Pulmonata
Opisthobranchia
Heterostropha
Pulmonata
Caenogastropoda
0.1

*Roboastra europaea**	SLLIRFELGTAGAFL**G**DDHFYNVIVTAHAFVMIFFMVMPLMI	Opisthobranchia
*Pupa strigosa**	SLLIRFELGTAGALL**G**DDHFYNVIVTAHAFVMIFFMVMPLMI	
Chelidonura africana	SLLIRFELGTASAFL**G**DDHFYNVIVTAHAFVMIFFMVMPLMI	
Aplysia punctata	SLLIRFELGTAGAFL**G**DDHFYNVIVTAHAFVMIFFMVMPIMI	
Umbraculum mediterraneum	SLLIRFELGTAGAFL**G**DDHFYNVIVTAHAFVMIFFMVMPMMI	
Berthella plumula	SLLIRFELGTSGALL**G**DDHFYNVIVTAHAFVMIFFMVMPLMI	
Aeolidia papillosa	SLLIRFELGTAGALL**G**DDHLYNVIVTAHAFVMIFFMVMPLMI	
Ascobulla fragilis	SLLIRFELGTSGAFL**G**DDHFYNVIVTAHAFVMIFFMVMPLMI	
Siphonaria pectinata	SLLIRFELGTAGAFL**G**DDHFYNVIVTAHAFVMIFFMVMPLMI	Basommatophora (Pulmonata)
*Siphonaria zelandica**	SMLIRLELGTAGVML**G**DPHLYNVIVTSHAFVMIFFLVMPAMI	
*Salinator solida**	SLLIRFELGTAGVLM-DEHFYNVIVTAHAFVMIFFMVMPLMI	Basommatophora (Pulmonata)
*Onchidium sp.**	SLLIRFELGTAGVLL-DDHFYNVIVTAHAFVMIFFMVMPLMI	Systelommatophora (Pulmonata)
Onchidella celtica	SLLIRFELGTVGVLL-DNHFYNVIVTAHAFVMIFFMVMPLMI	
Helix aspersa	SWLIRFELGTSGVLT-DDHFYNVIVTAHAFVMIFFMVMPIMI	Stylommatophora (Pulmonata)
*Cepaea nemoralis**	SLLIRLELGTAGVLT-DDHFYNVIVMYAHALYDLFMVMPIMI	
Elona quimperiana	SLLIRLELGTSGVLS-DDHFFNVIVTAHAFVMIFFMVMPIMI	
Rumina decollata	SLLIRLELGTAGVLT-DDHFFNVVVTAHAFVMIFFMVMPIMI	
*Albinaria coerulea**	SLLIRLELGTSGTLT-DDHFYNVIVTAHAFVMIFFMVMPIMI	
*Hedleyoconcha delta**	SLLVRLELGTAGVLL-DDHFFNVIVTAHAFVMIFFMVMPLMI	
Myosotella myosotis	SLLIRLELGTAGMLL-DDHLFNVIVTAHAFVMIFFMVMPLMI	Ellobioidea (Pulmonata)
*Ophicardelus ornatus**	SLLIRFELGTAGNLL-DDHFYNVIVTAHAFVMIFFMVMPLMI	
Pyramidella dolabrata	SLLIRYELGTAGVLT-DEHFYNVVVTAHAFVMIFFMVMPLMI	Pyramidelloidea (Heterostropha)
*Cornirostra pellucida**	SLLIRIELGTPGTFL**G**DDQLYNVIVTAHAFLMIFFFVMPMMM	Valvatoidea (Heterostropha)
*Cancellaria undulata**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	Caenogastropoda
*Conus miles**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	
*Mitra cucumerina**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	
*Dicathais orbita**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	
*Littorina saxatilis**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	
*Nassarius burchardi**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	
*Nerita atramentosa**	SLLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	Neritopsina
*Montfortula rugosa**	SMLIRAELGQPGALL**G**DDQLYNVIVTAHAFVMIFFLVMPMMI	Vetigastropoda
*Loligo bleekeri**	SLMIRTELGKPGTLL**N**DDQLYNVVVTAHGFIMIFFMVMPIMI	Cephalopoda

FIG. 5.—Indel event in the deduced amino acid sequence of the mitochondrial *cox1* gene of several gastropods. The indel is shown in bold and corresponds to position 46 in the Cox1 amino acid sequence of *R. europaea*. For opisthobranchs only representatives of the main lineages are shown. The following Cox1 amino acid sequences (denoted by an asterisk) were retrieved from GenBank: AY083457, NC_002179, AY296849, AY296845, AY296844, NC_001816, NC_001761, AY296871, AY296850, AY296842, AY296841, AY296840, AY296839, AY296838, AJ132137, AY296837, AY296824, AY296819, NC_002507.

gastropod systematics. The monophyly of both Opisthobranchia and Pulmonata, as well as that of Euthyneura, is clearly rejected (figs. 3 and 4). Opisthobranchia was recovered as a paraphyletic group because of the inclusion of the basommatophoran *S. pectinata*. The taxonomic position of the marine Siphonarioidea within the basommatophoran pulmonates has been discussed extensively (Köhler 1893; Haszprunar and Huber 1990; Nordsieck 1992). Haszprunar and Huber (1990) suggested the inclusion of Siphonarioidea in the basommatophoran, based on the symplesiomorphic presence of osphradium, pallial ciliary tracts, and several features of the nervous system (Tillier, Masselot, and Tillier 1996). Recent molecular studies based on 18S and 28S rRNA nuclear genes failed to recover Basommatophora as a monophyletic group (Tillier, Masselot, and Tillier 1996; Yoon and Kim 2000; Dutra-Clarke et al. 2001). In these studies, marine and freshwater basommatophorans are separated into clearly distinct groups that may be related to Opisthobranchia + Systelommatophora (in agreement with our results), and land Stylommatophora, respectively (Dutra-Clarke et al. 2001). In contrast, a recent study (Wade and Mordan 2000) based on a nuclear fragment including partial 5.8S rDNA, complete ITS-2, and partial large subunit rDNA sequences recovered *Siphonaria* as the sister group of other pulmonates. Hence, the phylogenetic status of *Siphonaria* (and Basommatophora in general) remains controversial.

The placement of the systelommatophoran *Onchidella* in a basal position relative to opisthobranchs independently rejected the monophyly of pulmonates

(figs. 3 and 4). Different morphologists have considered systelommatophorans as either opisthobranchs (Boettger 1955), pulmonates (Haszprunar 1988; Haszprunar and Huber 1990; Tillier and Ponder 1992) or even as an independent group closely related to opisthobranchs (Salvini-Plawen 1970).

The heterostrophan *Pyramidella* was recovered within the Euthyneura rendering this clade paraphyletic (figs. 3 and 4). In fact, pyramidellids have been considered by many authors to be opisthobranchs (Thorson 1946; Fretter and Graham 1949; Thompson 1973) because both groups share a rhinophoral nerve, a sinistral larval shell produced by the planktotrophic veliger (heterostrophy), simultaneous hermaphroditism, and the absence of a pectinibranch gill (ctenidium), among other characters. However, some authors criticized the validity of these characters as true synapomorphies (Gosliner 1981; Robertson 1985; Haszprunar 1988).

Interestingly, we found two mitochondrial RGCs, the relative position of the *trnP* gene and an indel in the Cox1 protein, that can be used as a valuable independent source to confirm and strengthen phylogenetic relationships within Heterobranchia (Euthyneura and heterostrophans) recovered from primary sequence data. The mitochondrial gene order is highly conserved in Heterobranchia with few tRNA gene translocations (Kurabayashi and Ueshima 2000*b*; Grande et al. 2002). Hence, the distinct relative position of the *trnP* gene in different taxa (fig. 3) seems a very promising phylogenetic marker. According to our results, the mitochondrial gene order rrnL, trnL(cun), trnA, trnP, nad6, and nad5 is associated with the Opisthobranchia + *Onchidella* + *Pyramidella* clade, and it might represent a molecular synapomorphy of these taxa. However, it is also likely that this gene order may be the ancestral state of Heterobranchia. The relative position of the mitochondrial *trnP* gene would need to be determined in more heterostrophans and pulmonates to discern between these two competing hypotheses. According to our results, a Glycine in position 46 of the Cox1 protein was present in Caenogastropoda and related basal gastropods (formerly included in Prosobranchia) and was further deleted in the ancestor of Euthyneura + *Pyramidella*. A reversal (or a convergence) due to structural constraints to the ancestral condition in gastropods (i.e., presence of Glycine) may have occurred in the ancestor of Opisthobranchia + *Siphonaria*. Alternatively, although less parsimonious, several independent deletions of the Glycine in different lineages of pulmonates (except *Siphonaria*) and the heterostrophan *Pyramidella* may also explain the pattern found with equal likelihood.

The phylogenetic hypothesis presented here corroborates the close relationships among all lineages of opisthobranchs (their monophyly is only rejected because of the relative position of *Siphonaria*), as previously suggested (Thiele 1931; Grande et al. 2002); yet it strongly rejects the validity of pulmonates as a natural group (against most morphological studies; e.g., Haszprunar and Huber [1990]; Dayrat and Tillier [2002]). These results stress the need of a thorough re-evaluation of the morphological characters that were used to define the monophyly of pulmonates, and they support the independent and recurrent evolution of the lung as the respiratory surface in gastropods. The recovered phylogeny provides a robust phylogenetic framework for many comparative studies involving this group and may allow a better understanding of evolutionary trends within gastropods.

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