The Complete Mitochondrial Genome of the Nudibranch *Roboastra europaea* (Mollusca: Gastropoda) Supports the Monophyly of Opisthobranchs

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The complete nucleotide sequence (14,472 bp) of the mitochondrial genome of the nudibranch *Roboastra europaea* (Gastropoda: Opisthobranchia) was determined. This highly compact mitochondrial genome is nearly identical in gene organization to that found in opisthobranchs and pulmonates (Euthyneura) but not to that in prosobranchs (a paraphyletic group including the most basal lineages of gastropods). The newly determined mitochondrial genome differs only in the relative position of the *trnC* gene when compared with the mitochondrial genome of *Pupa strigosa*, the only opisthobranch mitochondrial genome sequenced so far. *Pupa* and *Roboastra* represent the most basal and derived lineages of opisthobranchs, respectively, and their mitochondrial genomes are more similar in sequence when compared with those of pulmonates. All phylogenetic analyses (maximum parsimony, minimum evolution, maximum likelihood, and Bayesian) based on the deduced amino acid sequences of all mitochondrial protein-coding genes supported the monophyly of opisthobranchs. These results are in agreement with the classical view that recognizes Opisthobranchia as a natural group and contradict recent phylogenetic studies of the group based on shorter sequence data sets. The monophyly of opisthobranchs was further confirmed when a fragment of 2,500 nucleotides including the mitochondrial *cox1, rrnL, nad6,* and *nad5* genes was analyzed in several species representing five different orders of opisthobranchs with all common methods of phylogenetic inference. Within opisthobranchs, the polyphyly of cephalaspideans and the monophyly of nudibranchs were recovered. The evolution of mitochondrial tRNA rearrangements was analyzed using the *cox1+rrnL+nad6+nad5* gene phylogeny. The relative position of the *trnP* gene between the *trnA* and *nad6* genes was found to be a synapomorphy of opisthobranchs that supports their monophyly.

Introduction

Sea slugs and their relatives (Gastropoda: Opisthobranchia) are one of the most diverse and successful groups of mollusks. The 5,000 or so opisthobranch species currently recognized are classified into nine orders (Cephalaspidea, Acochlidea, Rhodopemorpha, Sacoglossa, Anaspidea, Notaspidea, Thecostomata, Gymnostomata, and Nudibranchia) (Rudman and Willan 1998). They are distributed throughout the world and occur in almost every marine environment. Regression of the shell, development of aposematic colorations, and acquisition of toxic defenses are general evolutionary trends within the group (Poulicek, Voss-Foucart, and Jeuniaux 1991). Traditionally, opisthobranchs have been recognized as a natural group and have been included within the gastropods together with prosobranchs and pulmonates (Thiele 1929–1935, pp. 1–1134). Prosobranchs (which used to include caenogastropodans, heterostrophans, retigastropodans and other related groups) are currently considered to be a paraphyletic group (Haszprunar 1988; Ponder and Lindberg 1997; Winnepenninckx et al. 1998; Yoon and Kim 2000) and represent the most basal lineages of gastropods, with pulmonates as the closest relatives of opisthobranchs (Spengel 1881; Fretter and Graham 1962, pp. 1–755; Morton 1979, pp. 1–264; Rudman and Willan 1998). The latter two groups have several synapomorphies and are commonly referred to as Euthyneura (Spengel 1881).

Several recent phylogenetic analyses based on morphological characters support the validity of the Euthyneura clade but tentatively reject the monophyly of both opisthobranchs and pulmonates (e.g., Haszprunar 1985; Salvini-Plawen and Steiner 1996; Ponder and Lindberg 1997) (fig. 1*A* and *B*). Depending on the phylogenetic analysis, different lineages of pulmonates are placed as sister groups of different orders of opisthobranchs (fig. 1). Additionally, the monophyly of some opisthobranch orders (e.g., Cephalaspidea and Notaspidea) has also been recently questioned (Schmekel 1985; Mikkelsen 1996; Wägele and Willan 2000). Apparently, the high degree of convergence or parallelism exhibited by many morphological characters of opisthobranchs (associated with reduction and loss of the shell and the mantle cavity; Gosliner and Ghiselin 1984; Gosliner 1985; Salvini-Plawen and Steiner 1996) has seriously complicated phylogenetic inferences within the group.

Molecular data can help in these cases by providing an independent set of characters. So far, most molecular studies on the phylogeny of opisthobranchs have been based on nuclear *18S* and *28S rRNA* sequence data (Tillier et al. 1994; Winnepenninckx et al. 1998; Wollscheid and Wägele 1999; Yoon and Kim 2000; Dayrat et al. 2001). These studies recovered poorly resolved phylogenetic trees in which the relationships of opisthobranch orders were unclear and the monophyly of opisthobranchs was rejected (fig. 1*C* and *D*). The lack of resolution of nuclear rRNA gene sequence data is likely because of an extensive rate heterogeneity among sites that significantly reduces the number of positions that are phylogenetically informative at any level of divergence (Olsen and Woese 1993). A recent phylogeny of

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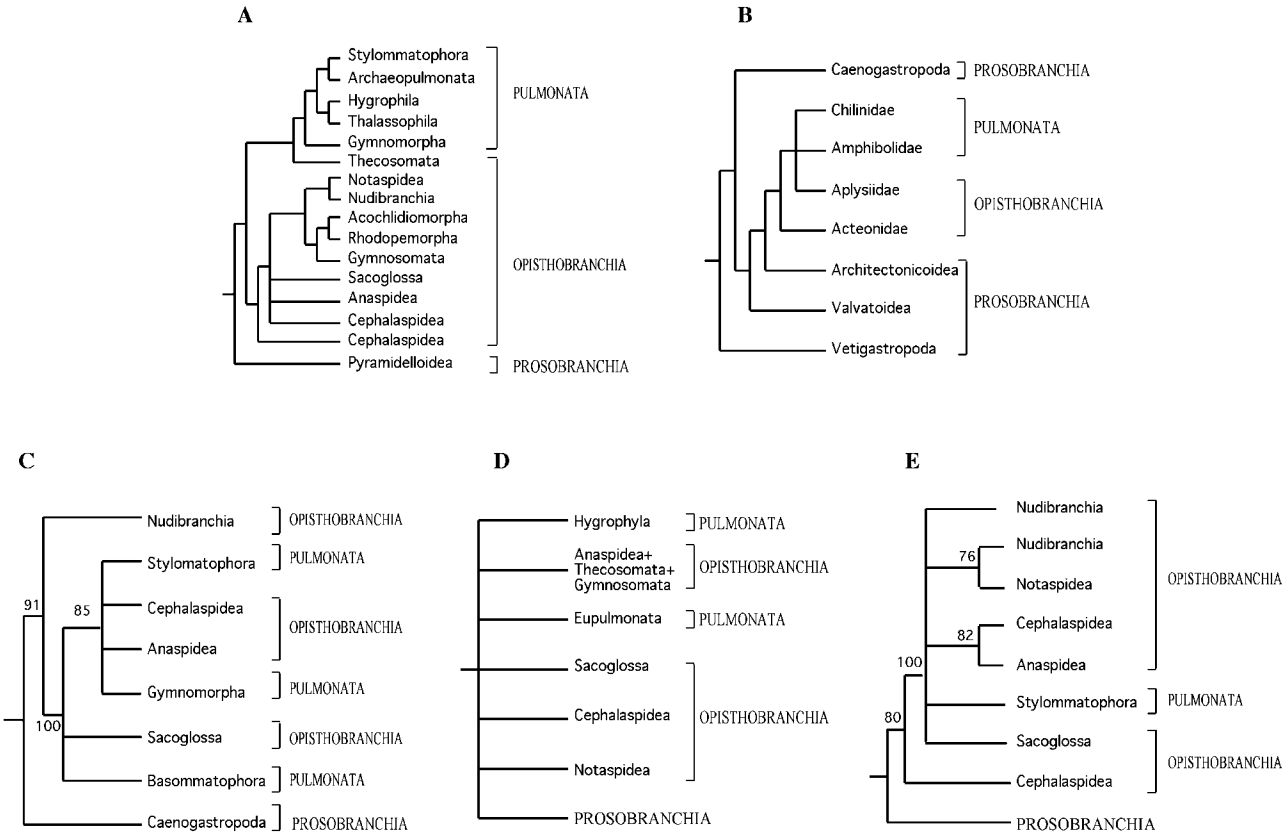

FIG. 1.—Phylogenetic hypotheses on the evolutionary position of opisthobranchs relative to other gastropods based on morphological ([*A*] Salvini-Plawen and Steiner 1996 and [*B*] Ponder and Lindberg 1997) and molecular data ([*C*] Wollscheid and Wägele 1999, [*D*] Dayrat et al. 2001, and [*E*] Thollesson 1999*b*). Note that all of them reject the monophyly of opisthobranchs. For the molecular phylogenies, nodes with bootstrap values below 50% were forced to collapse.

Euthyneura based on partial sequences of the mitochondrial *rrnL* gene rejected the monophyly of opisthobranchs as well as those of the orders Cephalaspidea and Nudibranchia (Thollesson 1999*b*) (fig. 1*E*). But these results were based on rather short sequence data. The phylogenies of some orders of opisthobranchs (Anaspidea and Nudibranchia) based on partial sequences of nuclear *18S rRNA* and mitochondrial *rrnS*, *rrnL*, and *cox1* genes were also published recently (Thollesson 1999*a;* Medina and Walsh 2000; Wollscheid et al. 2001).

Several phylogenetic analyses have demonstrated recently that the use of complete mitochondrial genomes in phylogenetic studies significantly increases the confidence of the phylogenetic history inferred compared with phylogenetic hypotheses based on individual or partial mitochondrial genes (Cummings, Otto, and Wakeley 1995; Russo, Takezaki, and Nei 1996; Zardoya and Meyer 1996). So far, the complete mitochondrial DNA sequences of seven mollusks are available: a cephalopod, *Loligo bleekeri* (Sasuga et al. 1999); a bivalve, *Crassostrea gigas* (S. H. Kim, E. Y. Je, and D. W. Park, personal communication; GenBank accession no. NC_001276); a polyplacophoran, *Katharina tunicata* (Boore and Brown 1994); and four gastropods—three pulmonates (*Albinaria coerulea* [Hatzoglou, Rodakis, and Lecanidou 1995], *Cepaea nemoralis* [Terrett, Miles, and Thomas 1996], and *Euhadra herklotsi* [Yamazaki et al. 1997]) and a primitive opisthobranch (*Pupa strigosa* [Kurabayashi and Ueshima 2000*a*]). The incomplete mitochondrial genomes of a bivalve (*Mytilus edulis* [Hoffmann, Boore, and Brown 1992]) and two gastropods (a caenogastropodan, *Littorina saxatilis* [Wilding, Mill, and Grahame 1999], and a heterostrophan, *Omalogyra atomus* [Kurabayashi and Ueshima 2000*b*]) have also been described.

The mitochondrial DNA of mollusks shows extreme variations in gene organization (Boore and Brown 1994; Yamazaki et al. 1997; Boore 1999; Kurabayashi and Ueshima 2000*a;* Rawlings, Collins, and Bieler 2001). The mitochondrial gene arrangements of pulmonates (*Euhadra*, *Cepaea*, and *Albinaria*), the heterostrophan (insofar as has been determined; *Omalogyra*), and the opisthobranch (*Pupa*) are nearly identical (Kurabayashi and Ueshima 2000*a*, 2000*b*). The gene organization of the mitochondrial genomes of *Littorina*, *Katharina*, and *Loligo* shows greater resemblance to the consensus gene arrangement of arthropods (Boore 1999; Sasuga et al. 1999; Wilding, Mill, and Grahame 1999). The lack of *atp8* gene in *Crassostrea* and *Mytilus* (Hoffmann, Boore, and Brown 1992; S. H. Kim, E. Y. Je, and D. W. Park, unpublished data; GenBank accession no. NC_001276), the presence of additional tRNA genes in *Katharina* and *Mytilus* (Hoffmann, Boore, and Brown

1992; Boore and Brown 1994), and an unusual mode of inheritance of *Mytilus* mitochondrial DNA (Zouros et al. 1994; Saavedra, Reyero, and Zouros 1997; Zouros 2000) are other intriguing features of the mitochondrial genomes of mollusks.

To test the monophyly of opisthobranchs and to clarify their relative phylogenetic position within gastropods, as well as to further investigate variations in the mitochondrial genome organization of mollusks, we have sequenced the complete mitochondrial genome of a nudibranch, *Roboastra europaea* García-Gómez 1985. We have compared this new mitochondrial genome with the only opisthobranch mitochondrial genome described so far, that of *P. strigosa*, and with mitochondrial genomes of other gastropods. To further understand opisthobranch systematics, we have also sequenced a mitochondrial DNA fragment of about 2,500 bp (including part of *cox1*, the complete *rrnL* and *nad6* genes, and a portion of the *nad5* gene) in several species that represent different orders of opisthobranchs.

Materials and Methods

DNA Extraction, Polymerase Chain Reaction Amplification, Cloning, and Sequencing

A single fresh specimen from Cabo de Trafalgar (Cádiz, Spain) was used to determine the sequence of the complete mitochondrial genome of *R. europaea*. Total cellular DNA was purified following a standard phenol-chloroform extraction. Universal primers were used to amplify fragments of the mitochondrial *rrnS* (H1478 and L1091; Kocher et al. 1989) and *rrnL* (16Sar-L and 16Sbr-H; Palumbi et al. 1991, pp. 1–45) genes by polymerase chain reaction (PCR). 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 30 cycles of denaturing at 94°C for 60 s, annealing at 42°C for 60 s, and extending at 72°C for 90 s. The amplified fragments were sequenced with the BigDye Deoxy Terminator cycle sequencing kit (Perkin-Elmer Biosystems) in an automated DNA sequencer (ABI PRISM 3100) using the PCR primers and following the manufacturer's instructions.

The sequences of both fragments were used to design two sets of specific primers (LP-F, LP1-R and LP1-F, LP-R; see table 1) that amplified, by long PCR, two fragments of about 7,000 bp each, that covered the remaining mitochondrial genome (fig. 2). Long PCRs containing 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 1–2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.4 μM of each primer, and elongase enzyme (1 unit; Life Technologies) in a final volume of 50 μl were subjected to 40 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 30 s, and extending at 68°C for 7 min.

Twenty-two more primers were designed on the basis of gastropod mitochondrial genome DNA sequences to amplify by standard PCR (see conditions described previously) overlapping fragments using the long PCR products as DNA templates (table 1 and fig. 2). The PCR products were cloned into the pGEM-T vector (Promega) and were sequenced using M13 universal primers (see conditions described previously). PCR amplification of a mitochondrial genome might introduce up to 0.25% mutations (Arnason, Xu, and Gullberg 1996). Divergences of 0.25% correspond to variation at the intraspecific level.

Total cellular DNA was also purified from several species of opisthobranchs that represent five different orders: *Chelidonura africana* (Cephalaspidea); *Ascobulla fragilis* (Sacoglossa); *Aplysia punctata* (Anaspidea); *Umbraculum mediterraneum* (Notaspidea); and *Aeolidia papillosa* (Nudibranchia). Two sets of primers, Opis COI-F (5′-ACTTTTTTTCCTCAGCATTTYTT-3′)/16Sbr and LP-F/Opis-2R (table 1), were used to amplify by standard PCR two overlapping DNA fragments that covered the 3′ end of the mitochondrial *cox1* gene, the complete mitochondrial *rrnL, trnL(cun), trnA, trnP,* and *nad6* genes, and the 5′ end of the mitochondrial *nad5* gene. PCR products were cloned into the pGEM-T vector (Promega) and were sequenced using M13 universal primers.

Molecular and Phylogenetic Analyses

Sequence data were analyzed with the GCG program version 10.2 (Devereux, Haeberli, and Smithies 1984), MacClade version 3.08a (Maddison WP and Maddison DR 1992, pp. 1–398), and PAUP* version 4.0b8 (Swofford 1998). Nucleotide and amino acid sequences were aligned using CLUSTAL X 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). Alignments are available from http://www.molbiolevol.org.

The following five complete mollusk mitochondrial genomes were analyzed in this study: *L. bleekeri* (Sasuga et al. 1999); *A. coerulea* (Hatzoglou, Rodakis, and Lecanidou 1995); *C. nemoralis* (Terrett, Miles, and Thomas 1996); *P. strigosa* (Kurabayashi and Ueshima 2000*a*); and *R. europaea* (this study). *Loligo bleekeri* was used as the out-group in all phylogenetic analyses because most authors currently consider cephalopods as the sister group of gastropods (Haszprunar 1988; Bieler 1992). The deduced amino acid sequences of all 13 protein-coding genes encoded by the mitochondrial genomes were combined into a single data set that was subjected to maximum parsimony (MP), minimum evolution (ME), maximum likelihood (ML), and Bayesian methods of phylogenetic inference. MP analyses were performed with PAUP* using heuristic searches (TBR branch swapping; MulTrees option in effect) with 10 random additions of taxa. ME analyses (Rzhetsky and Nei 1992) were carried out with PAUP* using mean character distances. ML analyses were performed with PUZZLE version 4.0.1 (Strimmer and von Haeseler 1996) using the mtREV model (Adachi and Hasegawa 1996). The robustness of the resulting MP and ME trees was evaluated by bootstrapping (Felsenstein 1985) (as implemented in PAUP* with 1,000 pseudoreplicates).

Table 1
Primers Used in the Sequencing of the Complete Mitochondrial Genome of *Roboastra europaea*

	Sequence (5′ → 3′)	Approximate Product Length (bp)
Long PCR primers		
LP-F	GTTTGTGACCTCGATGTTGGACT	7,400
LP1-R	GAAATTGGACTTGAAAGTAA	
LP-R	GATTGCGCTACCTTAGCACGGTCA	7,100
LP1-F	TCATTTAYCTGGTAAGTTCCCCT	
Standard PCR primers		
Opis1-F	GGGGATGATCACTTTTATAATGT	2,080
Opis1-R	ATTAYGCTACCTTAGCACRGTCA	
16Sar-L[a]	CGCCTGTTTATCAAAAACAT	450
16Sbr-H[a]	CCGGTCTGAACTCAGATCACGT	
LP-F	See above	1,460
Opis2-R	ACTAGAGTAGAAGAGTGGACTAA	
Opis3-F	ACTAAAAGAGCCCAGTATCCTTT	830
Opis3-R	GAAAAACAAAGTTGATAGGGCTC	
Opis4-F	CTCGATTATGGTTGGTTAGAGCC	700
Opis4-R	TCCCCCTCTGCAAAATCAAA	
Opis5-F	TGGGCTTCAAACTCTAAATA	1,340
Opis5-R	GTAGCACCTCAAAAAGATATTTG	
Opis6-F	ATAGCAACAGCATTTGTGG	1,240
Opis6-R	TCGGGGATTATATAAGAATC	
Opis7-F	CACGCTAACGGAGCTTCGCTTTT	1,190
Opis7-R	ATTCAAGCCTATGTTTTTAC	
Opis8-F	GGTGTTAATTGTGGCAT	960
Opis8-R	AACATATACTGAATCTTTAATC	
H1478[b]	TGACTGCAGAGGGTGACGGGCGGTGTGT	340
L1091[b]	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	
LP1-F	See above	1,540
Opis9-R	ACATGAGCCTTAGGGAGCCA	
Opis10-F	TGAGGTTATCAGCCCGAACG	2,100
Opis10-R	GCTTGAATACAAAAATATTT	
Opis11-F	CGCCGGCGAACGGGTATCATTGATGT	1,250
Opis11-R	ACTATTCAATTTCCAAACCCCCCAAT	
Opis12-F	GGTCTACCCCCTTTTATTGTATT	1,980
Opis12-R	AACCATTATACAAAAGGTA	

[a] Palumbi et al. (1991).
[b] Kocher et al. (1989).

The robustness of the resulting ML tree was evaluated by quartet puzzling (as implemented in PUZZLE with 10,000 puzzling steps). A Bayesian inference of gastropod phylogeny was performed with MrBayes 2.01 (Huelsenbeck and Ronquist 2001) by simulating a Markov chain for 10,000 cycles under the Jones model (Jones, Taylor, and Thornton 1992). The same phylogenetic analyses at the amino acid level were performed including only the mitochondrial protein-coding genes that were available for the caenogastropodan *L. saxatilis* (Wilding, Mill, and Grahame 1999), i.e., *cox1*, *cox2*, *atp8*, *atp6*, *nad1*, *nad6*, and *cob*. Sequence data of the six protein-coding genes known for the heterostrophan *O. atomus* (Kurabayashi and Ueshima 2000*b*) were not included in the phylogenetic analyses because they were not available on EMBL-GenBank data libraries.

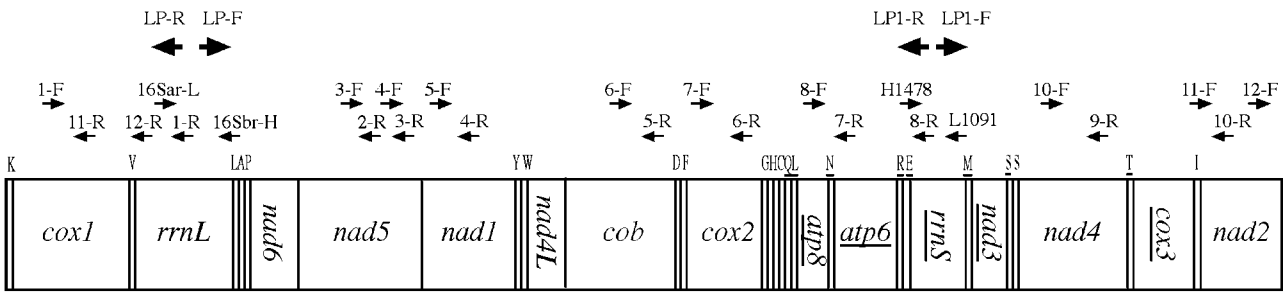

FIG. 2.—Gene organization and sequencing strategy for the mitochondrial genome of *R. europaea*. Genes encoded by the minor strand are underlined. Arrows denote the localization and direction of the primers used in the PCR amplification (see table 1 for the primer DNA sequence associated with each number).

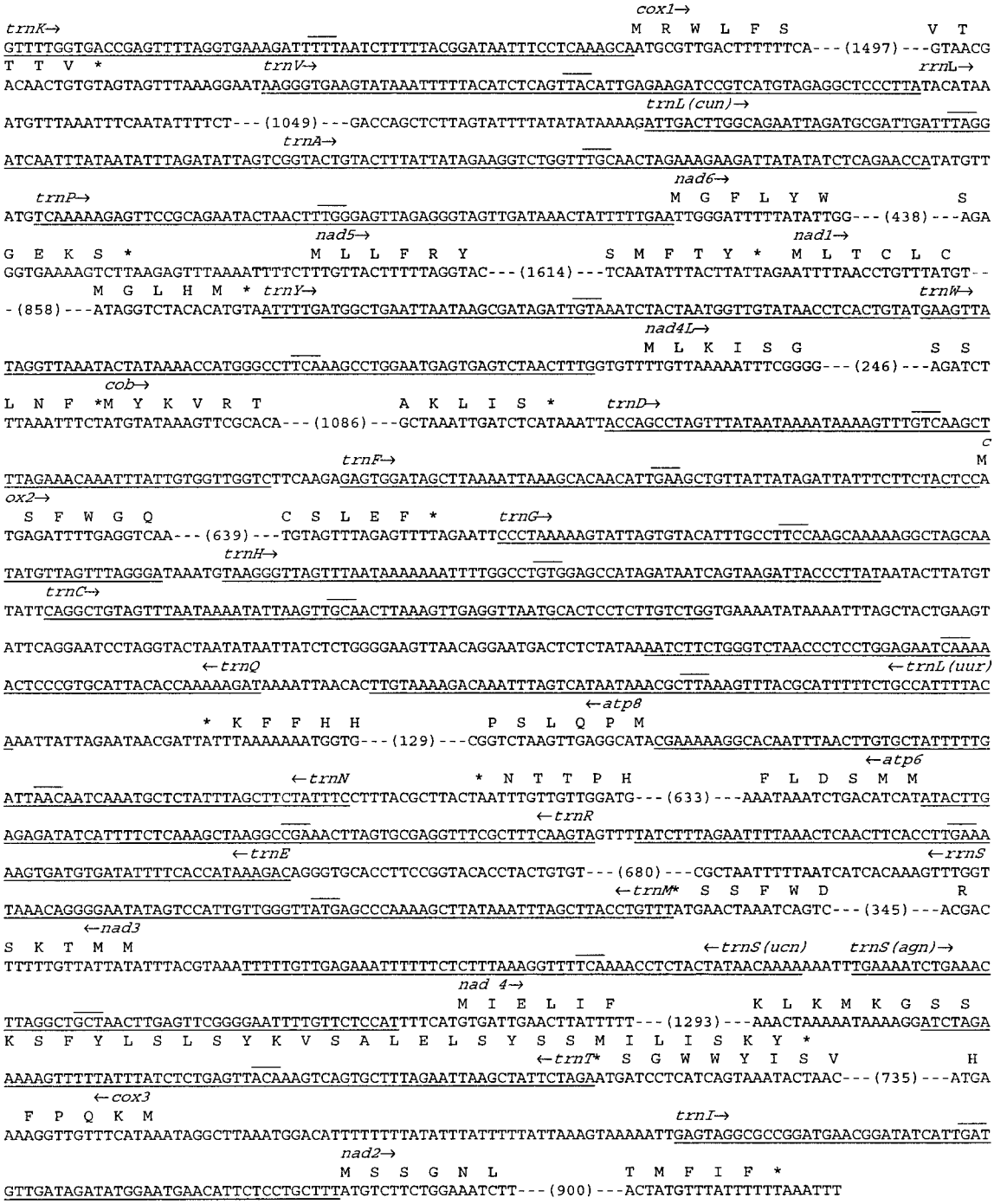

FIG. 3.—Schematic representation of the major strand nucleotide sequence of the mitochondrial genome of *R. europaea*. Position 1 corresponds to the first nucleotide of the *trnK* gene. Direction of transcription for each gene is represented by arrows. The beginning and end of the deduced amino acid sequence for each gene product is shown above the nucleotide sequence (one-letter amino acid abbreviation is placed above the first nucleotide of each codon). Termination codons are indicated by an asterisk. tRNA genes are underlined, and the corresponding anticodons are overlined.

To recover phylogenetic relationships among opisthobranchs, nucleotide sequences of part of the mitochondrial *cox1* gene, the complete mitochondrial *rrnL* and *nad6* genes, and a fragment of the mitochondrial *nad5* gene of several species that represent the main orders of opisthobranchs were analyzed with common methods of phylogenetic inference. MP analyses were performed with PAUP* without weighting based on the estimated empirical value (Ts/Tv = 0.71). ME and ML analyses were performed with PAUP* using the GTR model (Rodríguez et al. 1990). The robustness of MP, ME, and ML analyses was tested by bootstrapping with 1,000 pseudoreplicates. Bayesian inference of opisthobranch phylogeny was performed with MrBayes 2.01 (Huelsenbeck and Ronquist 2001) using the GTR model and 10,000 generations.

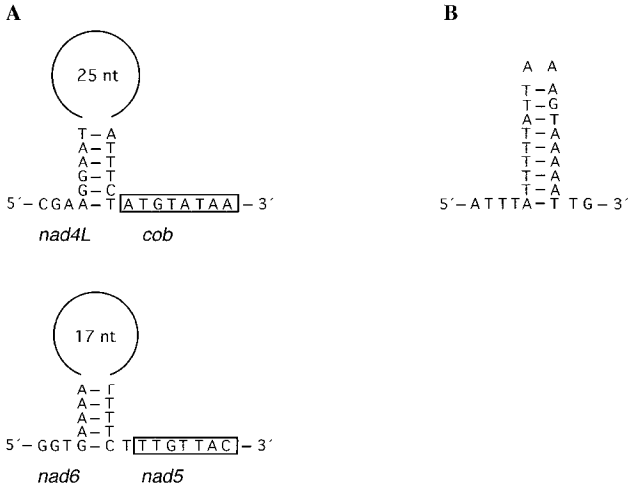

FIG. 4.—Putational functional secondary structures within the mitochondrial genome of *R. europaea*. *A*, Stem-loop structures between the end of the *nad4L* gene and the beginning of the *cob* gene, as well as between the *nad6* and *nad5* genes, which could serve as potential recognition signals for RNA-processing enzymes during transcription. *B*, A+T–rich hairpin structure between the *cox3* and *trnI* genes which potentially could be used as a signaling element for the control region of the *Roboastra* mitochondrial genome.

Statistical differences between alternative phylogenetic hypotheses were evaluated in PAUP* (Swofford 1998) using the Templeton (1983), Kishino and Hasegawa (1989), and Shimodaira and Hasegawa (1999) tests.

The nucleotide sequences of opisthobranchs reported in this article have been deposited at the EMBL-GenBank data libraries under accession numbers AY083457 and AY098927–AY098931.

Results

Genome Organization and Structural Features

The gene order and main features of the mitochondrial genome of *R. europaea* are summarized in figure 3. The total length of the mitochondrial DNA is 14,472 bp. The overall base composition of the major strand is A, 27.6%; T, 38.5%; C, 14.5%; and G, 19.4%. The mitochondrial genome of *R. europaea* encodes 2 rRNAs, 22 tRNAs, and 13 protein-coding genes (figs. 2 and 3). The major strand encodes 13 out of the 37 genes (*trnQ; trnL*(*uur*); *atp8; trnN; atp6; trnR; trnE, rrnS; trnM; nad3; trnS*(*ucn*), *trnT; cox3*). All protein-coding genes are separated by tRNA genes except two sets of genes (*nad6*/*nad5*/*nad1* and *nad4L*/*cob*). A potential stem-loop secondary structure which could putatively serve as a signal for RNA-processing enzymes (Boore and Brown 1994) was found between *nad4L* and *cob* genes, as well as between the *nad6* and *nad5* genes, but not between the *nad5* and *nad1* genes (fig. 4*A*). The largest noncoding regions are located between *trnC* and *trnQ* genes (93 bp) and between *cox3* and *trnI* genes (54 bp) (fig. 3). The latter region has the potential to fold into a hairpin secondary structure (fig. 4*B*).

The mitochondrial *rrnL* (1,109 bp) and *rrnS* (740 bp) genes are located between *trnV* and *trnL*(*cun*) genes and between *trnE* and *trnM* genes, respectively (figs. 2 and 3). The mitochondrial genome of *R. europaea* contains 22 tRNA genes that range in size from 54 to 69 nucleotides (fig. 5). All the deduced tRNAs can be folded into a cloverleaf secondary structure with the exception of tRNA(S-UCN) and tRNA(S-AGN) that lack the DHU arm. Mismatches in the acceptor stems of tRNA(L-CUN) and tRNA(Y) (fig. 5) might be corrected by the tRNA editing described by Yokobori and Pääbo (1995). Interestingly, the *trnC* gene is located between *trnH* and *trnQ* genes and not between *trnN* and *atp6* genes as in the mitochondrial genome of *Pupa* (Kurabayashi and Ueshima 2000*a*). The anticodons of all *Roboastra* mitochondrial tRNAs are preceded by a thymine and followed by a purine.

Seven out of the 13 protein-coding genes use ATG as the start codon (*cox1, cob, cox2, atp8, atp6, cox3,* and *nad2*). Other genes begin with TTG (*nad6, nad5,* and *nad4L*), ATA (*nad3*), ATT (*nad1*), and GTG (*nad4*) (fig. 3). Most *Roboastra* open reading frames end with TAA (*nad6, cob, atp8, nad4,* and *nad2*) or TAG (*cox1, nad5, cox2,* and *atp6*). The remaining have incomplete stop codons, either T (*nad4L, nad3,* and *cox3*) or TA (*nad1*) (fig. 3). In many mitochondrial genomes, *nad4L* has a complete stop codon and overlaps with a contiguous protein-coding gene. An alternative complete stop codon for *nad4L* can be postulated if it overlaps *cob* by eight nucleotides.

The genetic code of the *Roboastra* mitochondrial genome is the same as that used by other mollusks. It differs from the universal genetic code in that ATA codes for methionine, TGA for tryptophan, and AGR for serine. A total of 3,665 amino acids are encoded by the *Roboastra* mitochondrial genome (table 2). The most abundant amino acid residue is leucine, whereas the rarest is cysteine (table 2). Thymines are preferentially used in third codon positions. Cytosine is generally the rarest nucleotide in third codon positions (table 2).

Sequence Variation and Phylogenetic Analyses

The deduced amino acid sequences of all 13 mitochondrial protein-coding genes were combined into a single data set that produced an alignment of 3,869 positions. Of these, 1,227 were excluded from the analyses because of ambiguity in the homology assignment, 980 were invariant, and 357 were parsimony-informative. The mean character distance between *Roboastra* and *Pupa* is 0.27. The mean character distance between *Albinaria* and *Cepaea* is 0.43. The average mean character distance between opisthobranchs and pulmonates is 0.40.

ML analysis of the combined amino acid data set arrived at a tree (log likelihood = −22,320.08) that strongly supports the monophyly of opisthobranchs (*Pupa* + *Roboastra*) and pulmonates (*Albinaria* + *Cepaea*) (fig. 6). Bayesian inference rendered an identical result (fig. 6*A*). When MP (one single tree of 3,441 steps; consistency index [CI] = 0.94) and ME (score = 1.03) analyses were performed, only the opisthobranchs were recovered as a monophyletic group (fig. 6*A*). But

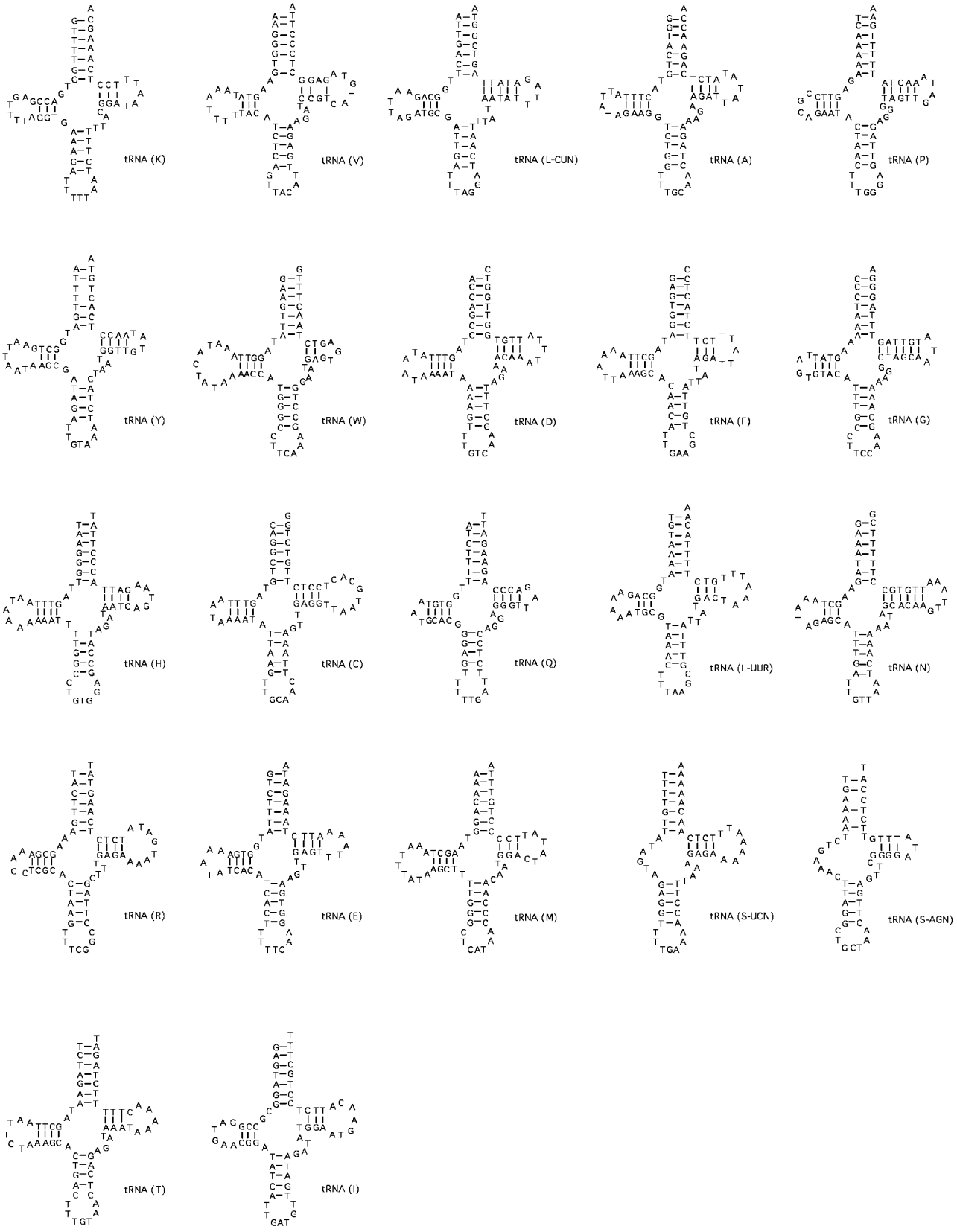

FIG. 5.—Proposed cloverleaf secondary structures of the 22 tRNAs deduced from the complete sequence of the mitochondrial genome of *R. europaea*.

Table 2
Codon Usage of *Roboastra europaea* Mitochondrial DNA–Encoded Proteins

Amino Acid	Codon	Number	Fraction	Amino Acid	Codon	Number	Fraction
Gly	GGG	83	0.29	Trp	TGG	40	0.44
Gly	GGA	86	0.30	Trp	TGA	50	0.56
Gly	GGT	104	0.36	Cys	TGT	45	0.82
Gly	GGC	14	0.05	Cys	TGC	10	0.18
Glu	GAG	43	0.47	End	TAG	4	0.31
Glu	GAA	49	0.53	End	TAA	9	0.69
Asp	GAT	58	0.85	Tyr	TAT	97	0.68
Asp	GAC	10	0.15	Tyr	TAC	45	0.32
Val	GTG	47	0.17	Leu	TTG	89	0.16
Val	GTA	73	0.26	Leu	TTA	265	0.47
Val	GTT	140	0.51	Phe	TTT	261	0.78
Val	GTC	17	0.06	Phe	TTC	72	0.22
Ala	GCG	18	0.08	Ser	TCG	24	0.06
Ala	GCA	39	0.18	Ser	TCA	63	0.16
Ala	GCT	126	0.58	Ser	TCT	116	0.30
Ala	GCC	34	0.16	Ser	TCC	13	0.03
Ser	AGG	33	0.09	Arg	CGG	15	0.26
Ser	AGA	55	0.14	Arg	CGA	20	0.34
Ser	AGT	65	0.17	Arg	CGT	18	0.31
Ser	AGC	18	0.05	Arg	CGC	5	0.09
Lys	AAG	28	0.29	Gln	CAG	14	0.27
Lys	AAA	67	0.71	Gln	CAA	37	0.73
Asn	AAT	74	0.78	His	CAT	46	0.58
Asn	AAC	20	0.22	His	CAC	33	0.42
Met	ATG	56	0.30	Leu	CTG	18	0.03
Met	ATA	128	0.70	Leu	CTA	60	0.11
Ile	ATT	220	0.87	Leu	CTT	98	0.18
Ile	ATC	32	0.13	Leu	CTC	26	0.05
Thr	ACG	15	0.08	Pro	CCG	21	0.14
Thr	ACA	61	0.32	Pro	CCA	31	0.21
Thr	ACT	93	0.50	Pro	CCT	83	0.56
Thr	ACC	19	0.10	Pro	CCC	12	0.08

a Wilcoxon signed-ranks test (Templeton 1983) showed that the second most parsimonious tree that supported the monophyly of pulmonates was not statistically significantly different (3,444 steps; $Z = -0.31$; $P = 0.75$). *Cepaea* exhibits a rather long branch, and its basal position in the MP and ME analyses might be an artifact attributable to long-branch attraction by the out-group (Felsenstein 1978).

To include the caenogastropodan *L. saxatilis* into the phylogenetic analyses, a smaller subset of protein-coding genes including *cox1*, *cox2*, *atp8*, *atp6*, *nad1*, *nad6*, and *cob* was analyzed with MP, ME, ML, and Bayesian methods of phylogenetic inference. The alignment of 1,789 positions was analyzed. Of these, 689 were excluded from the analyses because of ambiguity in the homology assignment, 495 were invariant, and 249 were parsimony-informative. All phylogentic analyses recovered a monophyletic opisthobranchia clade and *Littorina* as the most basal taxon of the in-group (fig. 6*B*). In MP (one single tree of 1,287 steps; CI = 0.91), ME (score = 0.94), and ML (log likelihood = −9,451.60) phylogenetic analyses, pulmonates were paraphyletic (fig. 6*B*). In Bayesian phylogenetic analyses, however, pulmonates were monophyletic with a 98% posterior probability (not shown).

The nucleotide sequences of the mitochondrial *cox1*, *rrnL*, *nad6*, and *nad5* genes were combined into a single data set that produced an alignment of 2,631 positions. Of these, 942 were excluded because of ambiguity in the homology assignment, 437 were invariant, and 900 were parsimony-informative. The mean pairwise uncorrected *p* distance among opisthobranchs is 0.32 ± 0.03. The minimum and maximum uncorrected *p* distances are between *Aplysia* and *Umbraculum* (0.25) and between *Pupa* and both *Roboastra* and *Aplysia* (0.36), respectively. The uncorrected *p* distance between *Albinaria* and *Cepaea* is 0.47. The mean pairwise uncorrected *p* distance between opisthobranchs and pulmonates is 0.43 ± 0.02. ML (log likelihood = −15,722.75) and Bayesian methods of phylogenetic inference arrived at identical topologies (fig. 7). ME (score = 2.65) only differed from the ML tree in that *Aplysia* was recovered as sister group of *Umbraculum* to the exclusion of *Chelidonura*. MP recovered two trees of 3,779 steps (CI = 0.62), one with the topology shown in figure 7 and the other supporting the monophyly of pulmonates. In all cases, opisthobranchs were monophyletic with strong statistical support (fig. 7). The Kishino and Hasegawa (1989) and Shimodaira and Hasegawa (1999) tests rejected statistical differences between the ML tree and a tree with a monophyletic pulmonate clade (log likelihood = −15,724.52; $P = 0.78$ and $P = 0.38$, respectively). Within opisthobranchs, cephalaspideans (*Pupa* and *Chelidonura*) were polyphyletic. *Pupa* was consistently the sister group of *Ascobulla* (order Sacoglossa), and both species were placed basal to the rest of opisthobranchs (fig. 7). *Chelidonura* was placed in a derived position either as sister group of *Aplysia* (order Anaspidea) (MP, ML, and Bayesian analyses) or as sister group of *Aplysia* + *Umbraculum* (order Notaspidea) (ME analyses). Because of the low bootstrap support, the relationships between *Chelidonura*, *Aplysia*, and *Umbraculum* remain unresolved. The nudibranchs (*Roboastra* and *Aeolidia*) were monophyletic (fig. 7).

Rearrangements of the *trnV*, *trnL*(*cun*), *trnA*, and *trnP* genes located between the *cox1*, *rrnL*, and *nad6* genes were analyzed by mapping the relative positions of these tRNA genes onto the recovered phylogeny (fig. 8). The *trnV* gene is located between the *cox1* and *rrnL* genes in all in-group taxa. *Cepaea* only presents the *trnL*(*cun*) and *trnA* genes between the *rrnL* and *nad6* genes. *Albinaria* presents the *trnP* gene between the *trnL*(*cun*) and *trnA* genes. With this data set, it is not possible to infer which tRNA genes were located between the *rrnL* and *nad6* genes in the ancestors of euthyneurans and of pulmonates. In all opisthobranchs, the *trnP* gene is located between the *trnA* and *nad6* genes (fig. 8). This relative position of the *trnP* gene seems to be a synapomorphy of opisthobranchs.

Discussion

The mitochondrial genome of *R. europaea* is one of the smallest known among Metazoa. The small size of the genome of *Roboastra* is the result of its compact gene organization, the absence of long noncoding re-

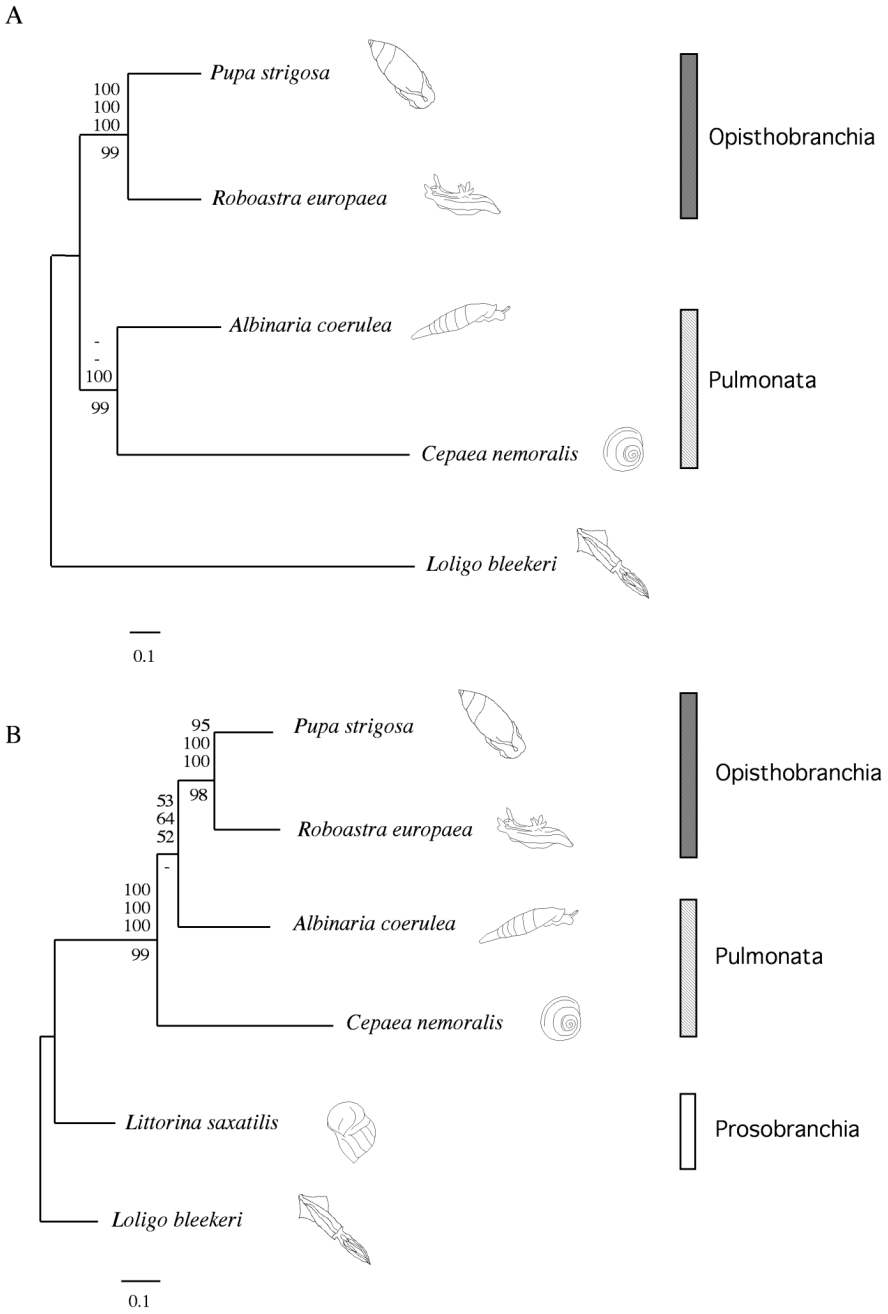

FIG. 6.—Phylogenetic position of *R. europaea*. *A*, The deduced amino acid sequences of all mitochondrial protein-coding genes of *Roboastra*, *Pupa*, *Albinaria*, and *Cepaea* were combined into a single data set. *B*, The deduced amino acid sequences of *cox1*, *cox2*, *atp8*, *atp6*, *nad1*, *nad6*, and *cob* genes of the aforementioned genera and *Littorina* were combined into a single data set. Both data sets were analyzed with MP (upper value of each triplet of numbers above branches represents bootstrap support), ME (mean character distances; middle value of each triplet of numbers above branches represents bootstrap support), ML (mtREV model; lower value of each triplet of numbers above branches represents quartet puzzling support), and Bayesian (Jones model; numbers below branches represent Bayesian posterior probabilities) methods of inference. The ML (mtREV model) trees are shown. *Loligo bleekeri* was used as the out-group.

gions, and the reduced size of its genes. This feature seems to be common to all Euthyneura because the size of the genome of *Roboastra* is within the range of variation in genome size found in *Pupa* (14.2 kb; Kurabayashi and Ueshima 2000*a*) and in pulmonates (14.1–14.5 kb; Hatzoglou, Rodakis, and Lecanidou 1995; Terrett, Miles, and Thomas 1996; Yamazaki et al. 1997). The mitochondrial genome of *Roboastra* shows an A+T content (66.1%) similar to that of other mollusks (Hoffman, Boore, and Brown 1992; Boore and Brown 1994; Hatzoglou, Rodakis, and Lecanidou 1995; Terrett, Miles, and Thomas 1996; Yamazaki et al. 1997; Sasuga et al. 1999; Kurabayashi and Ueshima 2000*a*, 2000*b*).

The gene arrangement of *Roboastra* is similar to that of *Pupa* (Kurabayashi and Ueshima 2000*a*) but differs in the transposition of the *trnC* gene. Moreover, the relative position of the *trnC* gene in pulmonates is different from that of both opisthobranchs (fig. 9). The re-

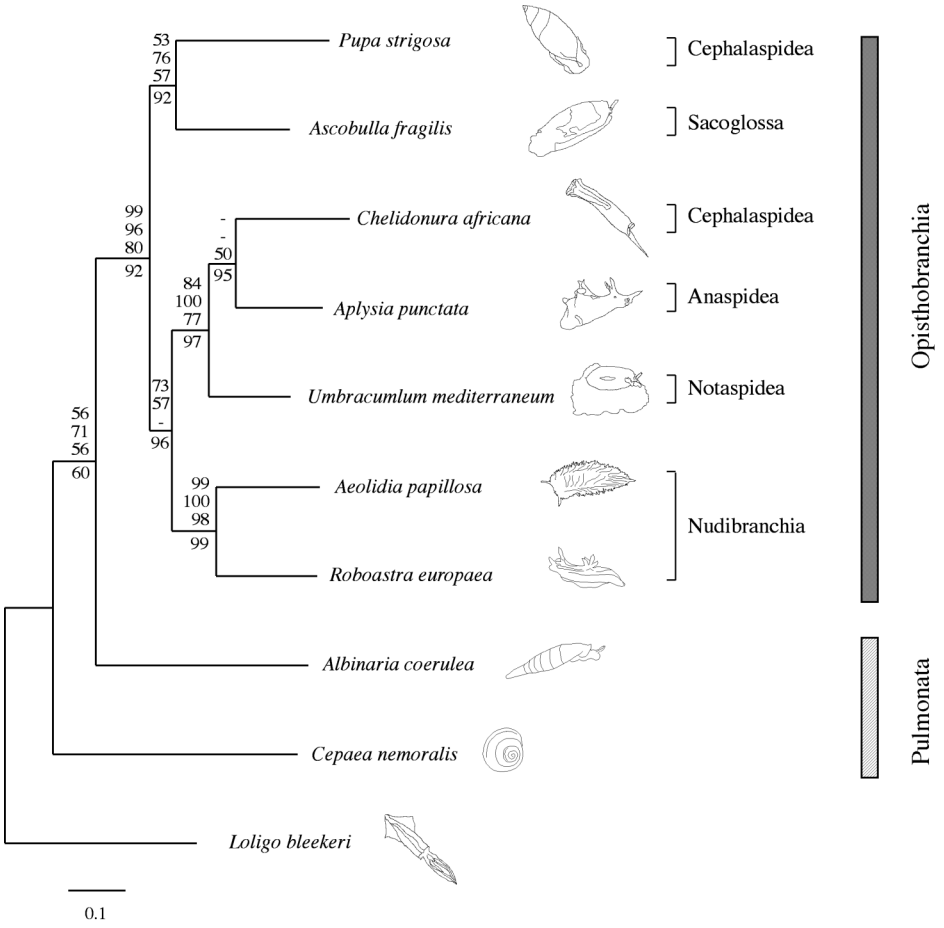

FIG. 7.—Phylogenetic relationships of representative orders of opisthobranchs as inferred from the nucleotide sequences of the *cox1*, *rrnL*, *nad6*, and *nad5* genes. The ML tree is shown. Numbers above branches represent MP (Ts:Tv = 1:1; upper value of each triplet of numbers), ME (GTR distances; middle value of each triplet of numbers), and ML (GTR model; lower value of each triplet of numbers) nonparametric bootstrap values. The numbers below branches represent Bayesian (GTR model) posterior probabilities. *Loligo bleekeri* was used as the outgroup.

arrangement of tRNA genes is very frequent in invertebrate mitochondrial genomes and may mobilize adjacent protein-coding and rRNA genes (Boore 1999). The Heterostropha, Pulmonata, and Opisthobranchia mitochondrial genomes described so far share a rather conserved gene arrangement (Hatzoglou, Rodakis, and Lecanidou 1995; Terrett, Miles, and Thomas 1996; Yamazaki et al. 1997; Kurabayashi and Ueshima 2000*a*, 2000*b*) (fig. 9). In contrast, a group traditionally considered within Prosobranchia, Caenogastropoda, shows a highly divergent gene arrangement that could be related to the gene arrangement of nongastropod mollusks (Boore 1999) (fig. 9). Therefore, the conserved gene arrangement of heterostrophans, pulmonates, and opisthobranchs (all together Heterobranchia) may present a derived state with respect to the ancestral state represented by the caenogastropodan *Littorina* (Boore 1999).

Of the two largest noncoding regions, that located between the *cox3* and *trnI* genes is the only one that contains a sequence with the potential to fold into a hairpin structure (fig. 4*B*). In addition, this region shows a high A+T content (87%). Both features are typical of invertebrate mitochondrial control regions (e.g., *Drosophila*; Clary and Wolstenholme 1985). Hence, this region seems to be a good candidate to contain the signals associated with the origin of replication and transcription of the *Roboastra* mitochondrial genome.

The amino acid sequence divergence between *Pupa* and *Roboastra* is almost half that between *Albinaria* and *Cepaea*. Furthermore, the amino acid sequence divergence within pulmonates is as much as that found between pulmonates and opisthobranchs. All phylogenetic analyses based on the combined protein-coding gene data set strongly support the monophyly of opisthobranchs (*Roboastra* and *Pupa*) (fig. 6*A*). The monophyly of opisthobranchs is also highly supported when *Littorina* is included in the phylogenetic analyses (fig. 6*B*). The genus *Pupa* is a representative of the most ancestral order (Cephalaspidea) of opisthobranchs. In fact, members of its family (Acteonidae) have been proposed as a model of the archetypal opisthobranch because their external morphology is similar to that of prosobranchs (Rudman 1972). In contrast, the genus *Roboastra* represents the most derived order (Nudibranchia) of the group with numerous morphological innovations (Wägele and Willan 2000).

Our results are in full agreement with the traditional view that opisthobranchs are a natural group of gas-

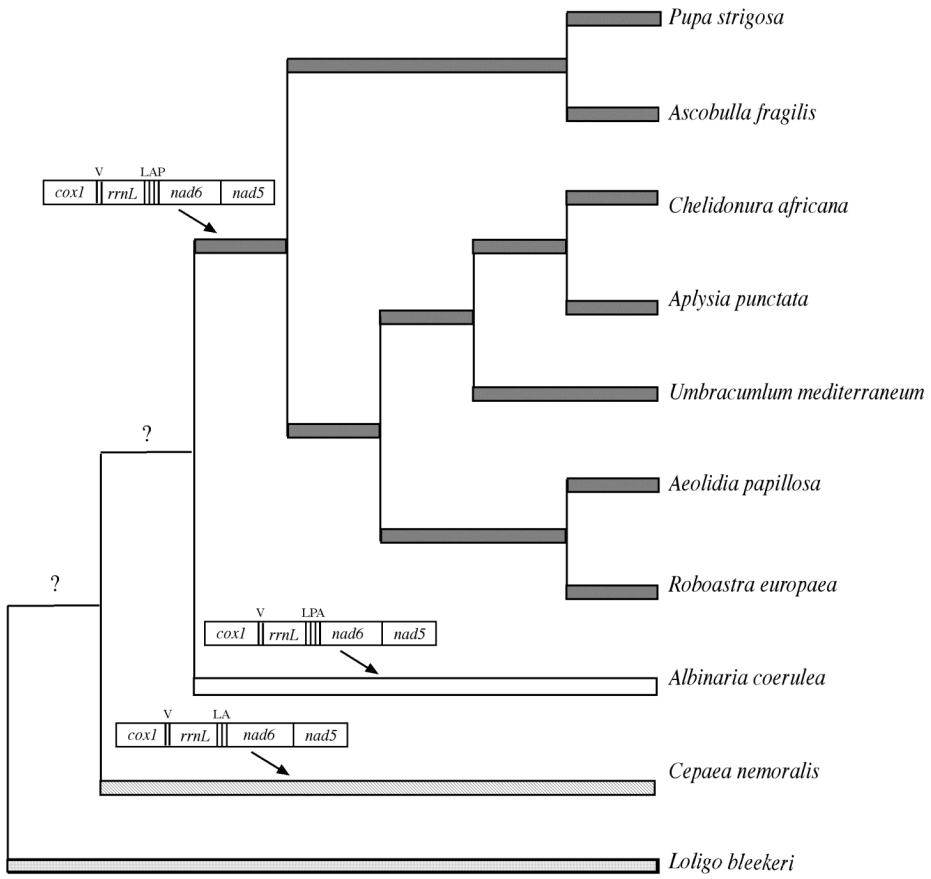

FIG. 8.—Mitochondrial tRNA gene rearrangements during gastropod evolution. The relative positions of the *trnV*, *trnL*(*UCN*), *trnA*, and *trnP* genes were mapped onto the *cox1*+*rrnL*+*nad6*+*nad5* phylogeny.

tropods (Thiele 1929–1935) and contradict recent molecular studies (e.g., Thollesson 1999*b;* Wollscheid and Wägele 1999; Dayrat et al. 2001) (fig. 1*C–E*). Our larger sequence data set and the lack of phylogenetically informative sites of previous molecular data sets (either because of their shorter size or their higher among-site rate variation) likely explain the discrepancy in resolution and statistical support between our results and those of previous studies. To test the validity of morphological hypotheses (Salvini-Plawen and Steiner 1996; Ponder and Lindberg 1997; fig. 1*A* and *B*) that challenge the monophyly of opisthobranchs, more opisthobranch as well as heterostrophan and pulmonate taxa need to be included in future phylogenetic analyses based on molecular data.

The monophyly of opisthobranchs is further supported by all phylogenetic analyses based on mitochondrial *cox1*, *rrnL*, *nad6*, and *nad5* genes at the nucleotide level (fig. 7). The relative position of the *trnP* gene between the *trnA* and *nad6* genes seems to be a shared derived character of opisthobranchs. Within opisthobranchs, the monophyly of cephalaspideans is rejected because *Pupa* is closely related to *Ascobulla* (order Sacoglossa), and *Chelidonura* appears as sister group to either *Aplysia* (order Anaspidea) or *Aplysia*+*Umbraculum* (fig. 7). This result is consistent with recent phylogenetic analyses based on morphological (Mikkelsen 1996) and molecular (Thollesson 1999*b*) data. According to morphological and molecular evidence, cephalaspideans can be separated into at least two groups: one basal to other opisthobranchs and the other related to anaspideans (Mikkelsen 1996; Thollesson 1999*b*). Our results only differ from these hypotheses in suggesting a close relationship of sacoglossans to basal cephalaspideans. This relationship is not that surprising because *Ascobulla*, which exhibits typical sacoglossan-type radular teeth, shares many external morphological characters with cephalaspideans (Mikkelsen 1998). The order Notaspidea is represented in our molecular phylogeny by the genus *Umbraculum* which is placed close to *Aplysia* and *Chelidonura* (fig. 7). This result supports previous morphological evidence that related some lineages of notaspideans to anaspideans (Schmekel 1985). The order Notaspidea includes two distinct groups, Umbraculomorpha and Pleurobranchomorpha (Rudman and Willan 1998). Several morphological characters such as an open seminal groove, a nonretractile penis, an albumen gland, plates in the gizzard, and the absence of a blood gland suggest that the Umbraculomorpha are closer to the Anaspidea than to the Pleurobranchomorpha (Schmekel 1985). Furthermore, the latter group seems to be more closely related to nudibranchs (Schmekel 1985; Wägele and Willan 2000). In this regard, a recent molecular phylogeny based on mitochondrial *rrnL* gene sequence data suggests that Pleurobranchomorpha may even be placed deep within the nudibranchs (Thollesson

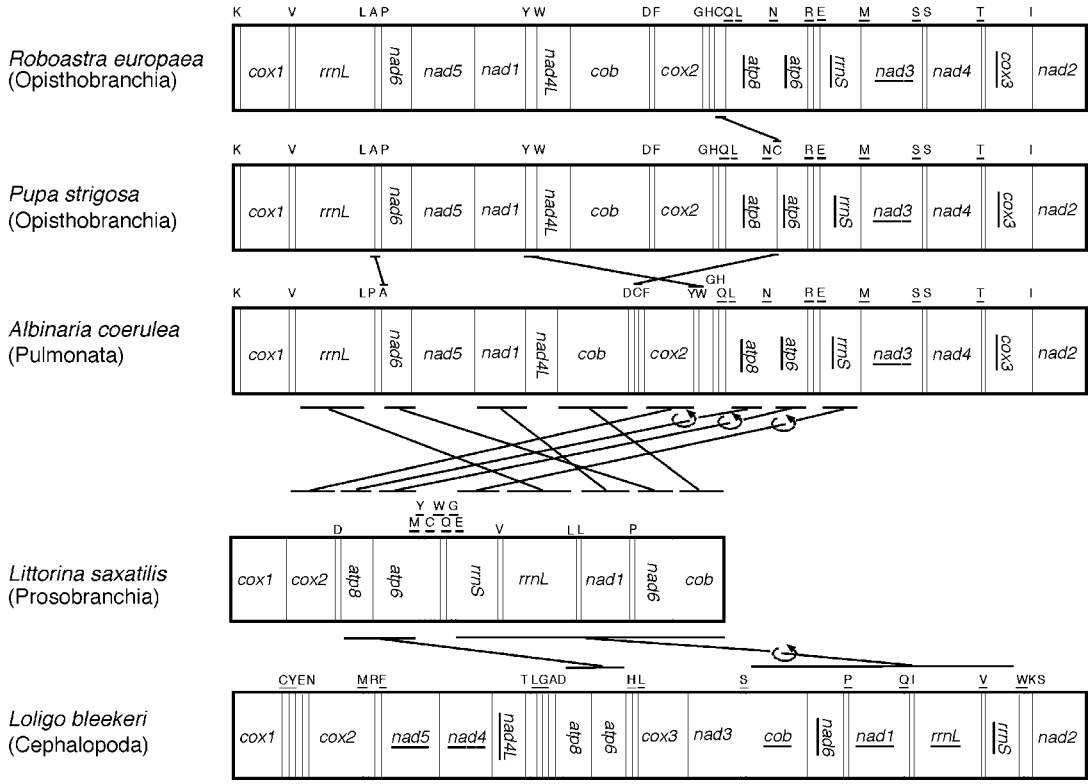

FIG. 9.—Differences in gene arrangement between *Roboastra* and other mollusks. Transpositions of protein-coding and rRNA genes are depicted among all taxa. Translocations of tRNA genes are only depicted among opisthobranchs and pulmonates. Most gene translocations have occurred between *Littorina* and Euthyneura (opisthobranchs and pulmonates). Genes encoded by the minor strand are underlined.

1999*b*). The monophyly of nudibranchs (without considering Pleurobranchomorpha) is well supported by several morphological (Schmekel 1985; Haszprunar 1988; Salvini-Plawen and Steiner 1996; Wägele and Willan 2000) and molecular (Wollscheid and Wägele 1999; Wollscheid et al. 2001) studies. The two nudibranchs (*Roboastra* and *Aeolidia*) included in our study are grouped together in the recovered phylogeny.

In conclusion, our analyses show that the newly determined mitochondrial genome of the nudibranch *R. europaea* is similar in size and gene arrangement to the mitochondrial genome of the cephalaspidean *P. strigosa*. Both species represent the most derived and basal lineages of opisthobranchs, respectively. These two mitochondrial genomes show the greatest sequence similarity when compared with other gastropod mitochondrial genomes. All phylogenetic analyses performed in this study both at the amino acid (including two orders) and at the nucleotide (including five orders) level, as well as the relative position of the *trnP* gene, support the monophyly of opisthobranchs with respect to pulmonates. The monophyly of this latter group is supported by ML and Bayesian analyses and is not rejected by MP analyses. Nevertheless, more representatives of heterostrophans, opisthobranchs, and pulmonates need to be included in future analyses to confirm further the monophyly of these gastropod groups.

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