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Molecular Phylogenetics and Evolution 33 (2004) 378–388

MOLECULAR PHYLOGENETICS AND EVOLUTION

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Phylogenetic relationships among Opisthobranchia (Mollusca: Gastropoda) based on mitochondrial *cox 1*, *trnV*, and *rrnL* genes

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Received 16 December 2003; revised 16 March 2004
Available online 12 August 2004

Abstract

We reconstructed the phylogenetic relationships among 37 species representing seven main lineages within Opisthobranchia (Mollusca: Gastropoda) based on a mitochondrial fragment that included partial *cox 1*, complete *trnV*, and partial *rrnL* genes (about 2500 bp). Phylogenetic analyses confirmed tentatively that all studied main opisthobranch lineages conformed monophyletic groups except Nudibranchia. The sacoglossan *Ascobulla* was placed as the most basal lineage of opisthobranchs. The basommatophoran pulmonate *Siphonaria* was recovered within Opisthobranchia between *Ascobulla* and the remaining opisthobranchs. The latter were divided into two different lineages that await formal description: on one side, Cephalaspidea, Tylodinoidea, and Anaspidea (sharing features in the reproductive, digestive, and circulatory systems) were grouped together and, on the other Architectibranchia and Nudipleura (sharing similarities in the circulatory system) were recovered as sister group taxa. Two well-supported clades were recovered within Nudipleura: Pleuroanthobranchia (new taxon) and Cladobranchia. Pleuroanthobranchia (Pleurobranchoidea plus Anthobranchia) was defined by the presence of blood gland, the presence of calcareous spicules in the integument and the presence of a caecum with an opening directly into the stomach. The new molecular phylogeny provided a robust framework for comparative studies, and prompted a revision of the morphological synapomorphies diagnosing the main clades within opisthobranchs.

Keywords: Opisthobranchia; mtDNA; Phylogeny; *cox 1*; *rrnL*; Gastropoda

1. Introduction

Opisthobranchs comprise structurally diversified and colourful organisms. They are cosmopolite, aquatic organisms that occupy a great variety of ecological niches, almost exclusively in marine habitats (Rudman and Willan, 1998). The main evolutionary trend of all the lineages within Opisthobranchia is the reduction or loss of the shell, which has allowed other body parts like the head, foot or mantle to become elaborated in diverse ways. The reduction of the shell and subsequent loss of physical protection have been accompanied by the evolution of other defensive strategies such as the acquisition of repugnatory glands and aposematic colorations.

Parallelism and convergence on morphological structures appear to have been commonplace during the radiation of opisthobranchs (Gosliner, 1991; Gosliner and Ghiselin, 1984; Mikkelsen, 1996), and hampered previous phylogenetic studies. As a result, several contradicting phylogenetic hypotheses and taxonomic classification systems of opisthobranchs were proposed through the years (Boettger, 1954; Ghiselin, 1966; Rudman and Willan, 1998; Taylor and Sohl, 1962; Thompson, 1976).

Opisthobranchs share several synapomorphies with pulmonates (another group of derived gastropods),

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1055-7903/$ - see front matter
doi:10.1016/j.ympev.2004.06.008

and together conform the clade Euthyneura (Spengel, 1881). However, the monophyly of opisthobranchs with respect to pulmonates remains unclear according to many phylogenenetic hypotheses based on morphological characters (Dayrat and Tillier, 2002; Ponder and Lindberg, 1997; Salvini-Plawen and Steiner, 1996). In a recent paper (Thollesson, 1999b) on the phylogenetic relationships of Euthyneura based on partial mitochondrial *rrnL* gene sequences data, stylommatophoran and basommatophoran pulmonates were recovered within opisthobranchs rendering the latter paraphyletic. In contrast, Grande et al. (2002) advocated for the monophyly of opisthobranchs based on phylogenetic analyses of partial mitochondrial *cox 1*, complete *rrnL*, complete *nad6*, and partial *nad5* gene sequence data. However, the authors noted that in that study pulmonate lineages were underrepresented (Grande et al., 2002). A more recent study (Grande et al., 2004), including more pulmonates and the heterostrophan *Pyramidella*, based on mitochondrial sequence data showed that both Euthyneura and Pulmonata were not monophyletic, and that the basommatophoran pulmonate *Siphonaria* was recovered deep within the opisthobranchs, rendering the latter paraphyletic.

There are 11 main groups (Architectibranchia, Cephalaspidea, Acochlidiomorpha, Rhodopemorpha, Anaspidea, Sacoglossa, Thecosomata, Gymnosomata, Tylodinoidea, Pleurobranchoidea, and Nudibranchia) currently recognized within Opisthobranchia (Mikkelsen, 1996, 2002; Rudman and Willan, 1998; Schmekel, 1985). Although many morphological and molecular phylogenetic studies have focused on some of these groups (Jensen, 1996; Medina et al., 2001; Medina and Walsh, 2000; Mikkelsen, 1996, 2002; Salvini-Plawen, 1970, 1991; Schmekel, 1985; Wägele and Willan, 2000; Willan, 1987; Wollscheid et al., 2001; Wollscheid and Wägele, 1999), their sister-group relationships remain unresolved. Two members of the lineage Architectibranchia (*Ringicula* and *Acteon*) (Fretter and Graham, 1954; Gosliner, 1981; Morton, 1968) have been alternatively proposed as the most basal opisthobranchs and therefore as a model of an archetypal opisthobranch. Although the monophyly of several groups (Anaspidea and Sacoglossa) is generally accepted (Jensen, 1996; Medina and Walsh, 2000; Mikkelsen, 1996, 2002; Schmekel, 1985; Thollesson, 1999b) the validity of others (Architectibranchia, Cephalaspidea, and Nudibranchia) is controversial (Mikkelsen, 1996, 2002; Minichev, 1970; Schmekel, 1985; Thollesson, 1999b; Wägele and Willan, 2000; Wollscheid et al., 2001; Wollscheid and Wägele, 1999). For instance, there are no morphological synapomorphies described for Architectibranchia (Mikkelsen, 2002). Regarding Nudibranchia, different phylogenetic hypotheses based on morphological and molecular data support them either as a monophyletic group (Boettger, 1954; Schmekel, 1985; Wägele and Willan, 2000; Wollscheid et al., 2001; Wollscheid and Wägele, 1999) or as paraphyletic group (Minichev, 1970; Thollesson, 1999b).

In this study, we have compiled partial sequences of the mitochondrial *cox 1* and *rrnL* genes as well as the complete sequence of the mitochondrial *trnV* gene (2500 bp) in several taxa representing seven out of the 11 groups of opisthobranchs. Mitochondrial genes have been shown to be useful in recovering phylogenetic relationships at different hierarchical levels among Opisthobranchia (Grande et al., 2002, 2004; Medina et al., 2001; Medina and Walsh, 2000; Remigio and Hebert, 2003; Thollesson, 1999a,b; Valdés, 2003; Wollscheid et al., 2001). Hence, they were expected to be useful for the phylogenetic question at hand. Primary sequences were analyzed with current methods of phylogenetic inference. The secondary structure of the *rrnL* gene (Lydeard et al., 2002) was also used to infer phylogenetic relationships among the studied taxa. Moreover, we review morphological synapomorphies that may support the different clades within the recovered molecular phylogenetic hypothesis.

2. Materials and methods

2.1. Taxon sampling and DNA extraction

Thirty-seven opisthobranchs and two pulmonates were analyzed in the present study (Appendix A). The heterostrophan *Pyramidella dolobrata* was used as outgroup. DNA was extracted from the foot except in those cases of small animals where the whole specimen was used. Tissues were grounded in liquid nitrogen and resuspended in 500 μl of extraction buffer (Towner, 1991). Total cellular DNA was isolated from each sample using phenol/chloroform extraction, and then precipitated with ethanol.

2.2. Polymerase chain reaction amplification, cloning, and sequencing

A fragment of about 2500 bp (including the partial sequences of the mitochondrial *cox 1* and *rrnL* genes and the complete sequences of *trnV* gene) was amplified by polymerase chain reaction (PCR) using four sets of primers: LCO-1490 and HCO-2198 (Folmer et al., 1994); OPISA-F and OPISA-R (Grande et al., 2004); OPIS COI-F and OPIS1-R (Grande et al., 2002), and 16Sar-L and 16Sbr-H (Palumbi et al., 1991).

Standard PCR reactions consisted of 40 cycles with a denaturing temperature of 94 °C for 60 s, annealing at 42–52 °C for 60 s, and extending at 72 °C for 90 s, in a total volume of 25 μl. PCR products were precipitated with ethanol, and either directly sequenced using the corresponding PCR primers, or cloned into the pGEM-T vector (Promega) and sequenced using 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) on an automated DNA sequencer (Applied Biosystems Prism 3700) following manufacturer's instructions.

2.3. Phylogenetic analyses

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). The nucleotide sequences of partial mitochondrial *cox 1* (only first and second positions) and *rrnL* genes, and the complete *trnV* gene were subjected to maximum parsimony (MP) and maximum likelihood (ML) analyses. MP was performed in PAUP* 4.0b10 (Swofford, 2002) using heuristic searches (MulTrees option in effect) with 10 random stepwise additions of taxa. A 3:1 transversion (Tv):transition (Ts) weighing scheme was used based on empirical evidence (Ts/Tv = 2.20). We used the Akaike information criterion (AIC) implemented in MODELTEST version 3.06 (Posada and Crandall, 1998) to determine the appropriate model of evolution. ML was performed in PAUP* using the GTR model (Rodriguez et al., 1990) and optimized parameter values. Robustness of MP and ML analyses was tested by bootstrapping with 1000 pseudoreplicates.

A combined data set that included the deduced partial amino-acid sequences of *cox 1* gene, the complete sequence of the *trnV*, and the partial nucleotide sequences of *rrnL* gene was analyzed using the mtREV (or mtREV+I+Γ) (Adachi and Hasegawa, 1996), and the GTR+I+Γ (Rodriguez et al., 1990) substitution models for amino acids and nucleotides, respectively ('set partition' and 'unlink' options) with Bayesian inference, the only available method of phylogenetic inference that can analyzed simultaneously partitions with different types of source data. Bayesian inference was performed using MrBayes 3.0b3 (Huelsenbeck and Ronquist, 2001) with random starting trees and run for 1,000,000 generations, sampling the Markov chains at intervals of 100 generations. Four heated Markov chains (using default heating values) were used. A total of 1000 out of the 10,000 resulting trees were discarded as "burn-in." To ensure that Markov chains were not trapped on local optima, Bayesian inferences were performed twice beginning with different starting trees, and apparent stationary levels were compared for convergence (Huelsenbeck and Bollback, 2001). Support for tree nodes was determined based on the values of Bayesian posterior probability (BPP) obtained from a majority-rule consensus tree.

3. Results

Phylogenetic relationships among opisthobranchs were reconstructed based on two different sequence data sets: one included nucleotide sequences of mitochondrial partial *cox 1* (only first and second positions), complete *trnV*, and partial *rrnL* genes, whereas the other was a combined data set of the deduced amino-acid sequences of partial *cox 1* gene and the nucleotide sequences of the complete *trnV* and partial *rrnL* genes.

The first data set produced an alignment of 2297 positions. The high variability exhibited by the *trnV* and *rrnL* genes sequences hampered the assessment of homologous positions in different parts of their alignment and thus, 911 positions were excluded. A total of 853 positions were invariant, and 402 were parsimony-informative. MP analyses arrived at one most-parsimonious tree of 4788 steps when a 3:1 Tv:Ts weighting was assumed (CI=0.32; RI=0.68) (Fig. 1). The systelommatophora *Onchidella* was the most basal ingroup lineage. The sacoglossan *Ascobulla* was the next lineage branching off the tree (Fig. 1). The basommatophoran pulmonate *Siphonaria* was recovered within Opisthobranchia (Fig. 1). The recovered topology showed two highly supported main lineages within opisthobranchs: one included Cephalaspidea, Anaspidea, and Tylodinoidea whereas the other included Architectibranchia and Nudipleura (Nudibranchia + Pleurobranchoidea) (Fig. 1). The monophyly of each of the groups within the two main lineages was supported by high bootstrap values (Fig. 1). However, the relative position of Pleurobranchoidea within Nudibranchia rendered the latter paraphyletic (Fig. 1). A general lack of resolution was observed within the terminal groups that was likely related with the high levels of saturation found in mitochondrial *cox 1* gene at the nucleotide level (not shown). ML (−ln L=13674.26) arrived to a tree with the same branching pattern (Fig. 1).

The second data set produced an alignment of 1797 positions. After removing all ambiguous positions (mostly in *trnV* and *rrnL* genes), a total of 886 positions were used for further phylogenetic analyses (392 nucleotide positions for *trnV* and *rrnL* genes and 494 inferred amino-acid positions for *cox 1* gene). Bayesian inferences based on this data set using the GTR+I+Γ (nucleotide sequence data) and the mtREV (amino acid sequence data) substitution models were performed. The reconstructed Bayesian 50% majority-rule consensus tree is depicted in Fig. 2. Alternatively, a Bayesian inference using the mtREV+I+Γ substitution model for the amino acid sequence data recovered the same tree and similar posterior probability values for all the nodes (not shown). The recovered topology supported the basal position of the order Sacoglossa (represented by *Ascobulla*) with respect to all other studied opisthobranchs (Fig. 2). The basommatophoran pulmonate *Siphonaria* was recovered

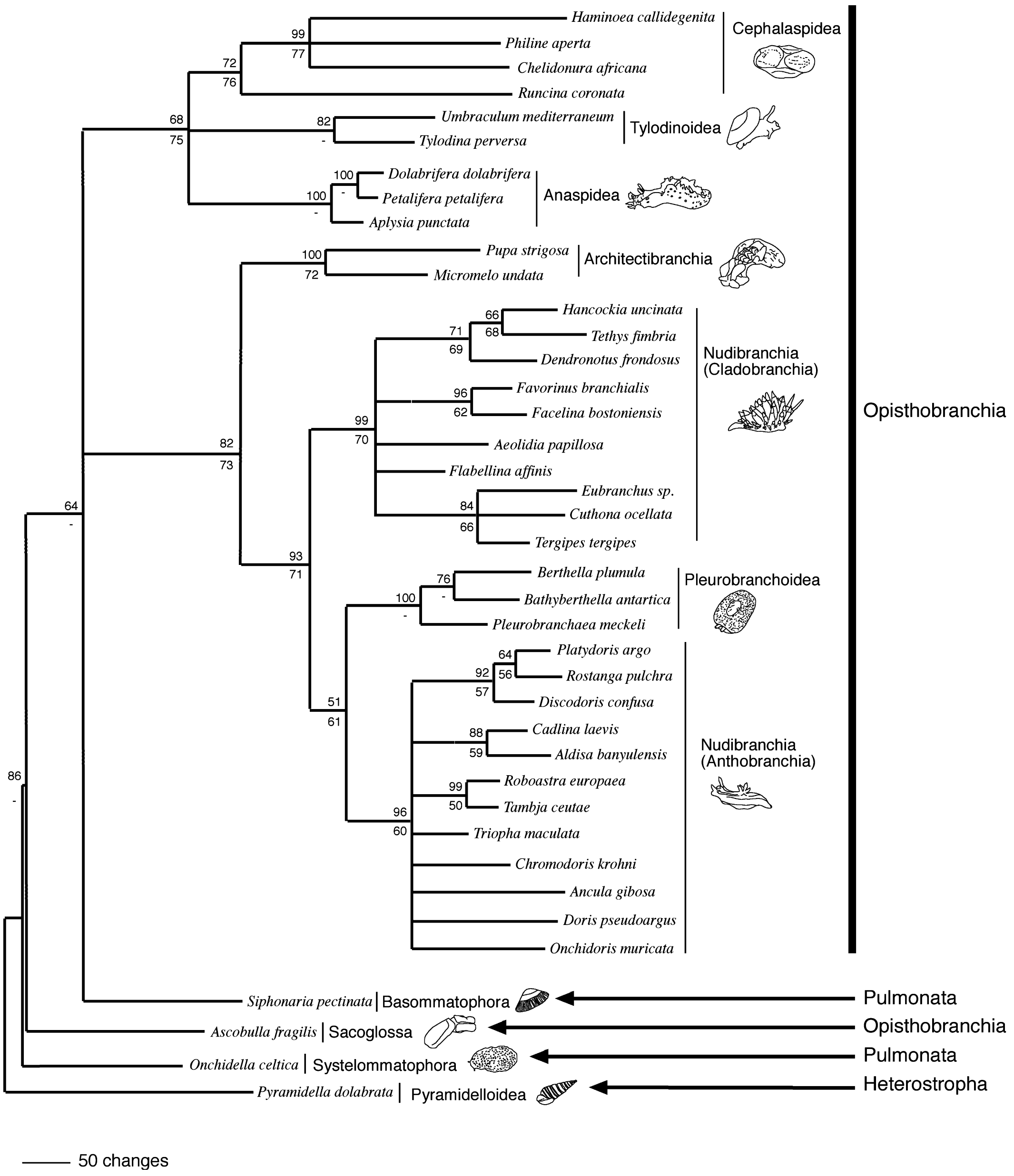

Fig. 1. Phylogenetic relationships of Opisthobranchia as inferred from the nucleotide sequences of the mitochondrial partial *cox 1* (only first and second positions), complete *trnV*, and partial *rrnL* genes. The MP tree is shown. The numbers above and below branches are bootstrap values corresponding to the MP (Tv:Ts = 3:1) and ML (GTR+I+Γ model) phylogenetic analyses, respectively. *Pyramidella* was used as outgroup.

within Opisthobranchia. The remaining studied opisthobranchs were resolved into two distinct lineages: one included Cephalaspidea, Anaspidea, and Tylodinoidea whereas the other included Architectibranchia and Nudipleura. Within Nudipleura, Pleurobranchoidea was recovered within Nudibranchia rendering the latter paraphyletic (Fig. 2). Higher support for the different inferred clades as well as higher resolution of terminal nodes were observed.

In addition to the phylogenetic analyses of primary sequence data, the secondary structure of *rrnL* mitochondrial gene was explored for all the studied taxa (Fig. 3) in order to search for any phylogenetically informative signal (Lydeard et al., 2002). We inferred the three helical–loop structures (within Domains II, III, and V of the *rrnL* mitochondrial gene) that were previously used as phylogenetically informative characters in a recent study of Heterobranchia (a stem of gastropods that includes Euthyneura + Heterostropha) (Lydeard et al., 2002). No helical–loop structures were found within Domains II and V in any of the studied taxa. Interestingly, members of the Nudipleura clade (Cladobranchia, Pleurobranchoidea, and Anthobranchia) shared a helical–loop structure within Domain

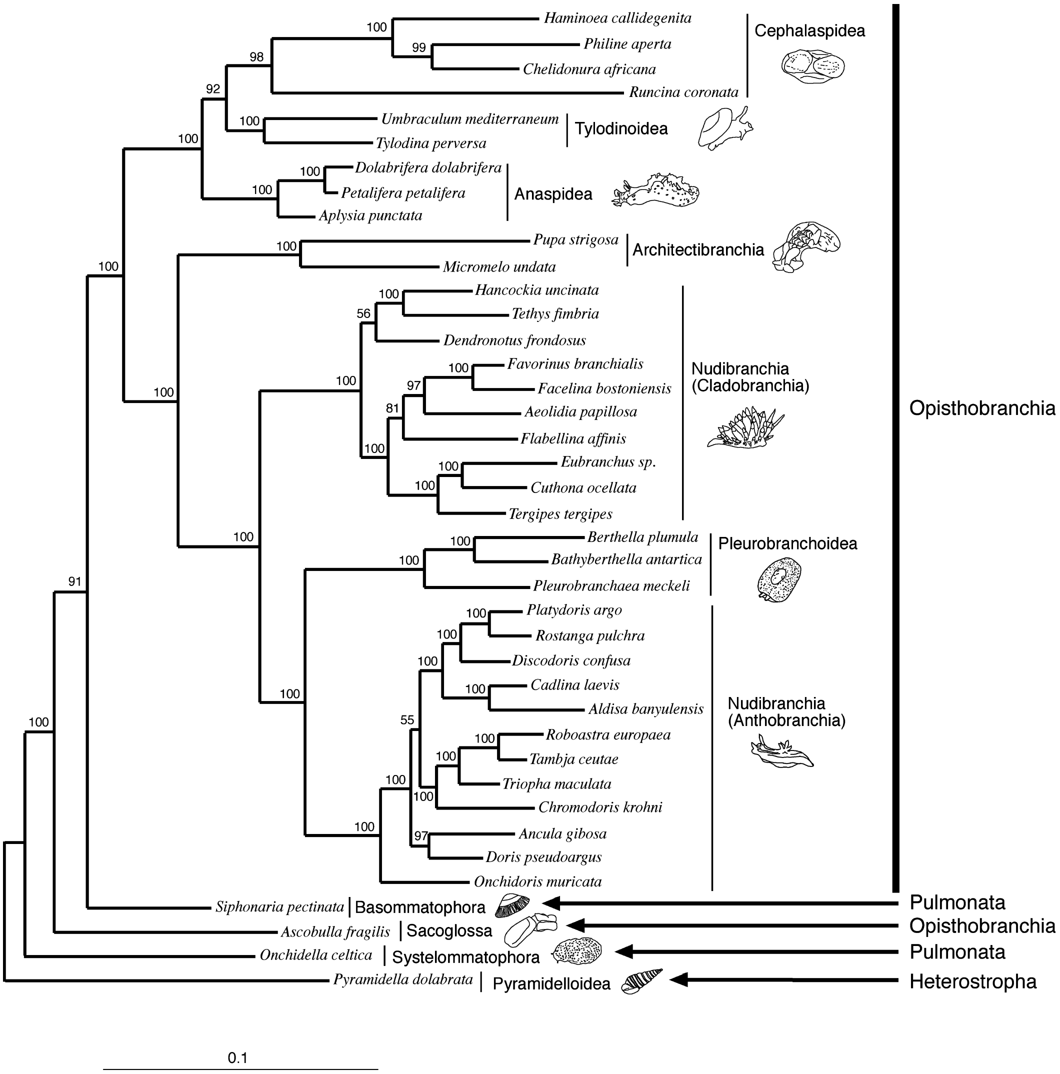

Fig. 2. Bayesian 50% majority rule consensus tree inferred from the deduced amino acid sequences of the mitochondrial partial *cox 1* gene (mtREV) and the nucleotide sequences of the mitochondrial complete *trnV*, and partial *rrnL* gene (GTR+I+Γ). The numbers above branches represent Bayesian posterior probabilities (only values above 95% are considered as statistically significant). *Pyramidella* was used as outgroup.

III of the *rrnL* secondary structure, which is absent in the other studied taxa (Fig. 3).

4. Discussion

The present study provides a robust phylogenetic hypothesis for the relationships among different lineages of opisthobranchs based on mitochondrial gene sequence data. Phylogenetic analyses of mitochondrial partial *cox 1* (only first and second positions), complete *trnV*, and partial *rrnL* gene nucleotide sequences reconstructed rather unresolved topologies, particularly at terminal nodes. However, the Bayesian analysis based on a combined data set including the deduced amino acid sequences of mitochondrial partial *cox 1* gene and the nucleotides sequences of mitochondrial complete *trnV* and partial *rrnL* genes arrived at a highly resolved tree that is our best hypothesis for the phylogenetic relationships of opisthobranchs. The difference in resolution between both phylogenetic trees was likely due to saturation of mitochondrial sequences at the nucleotide level (but not at the amino acid level) that resulted in an adverse phylogenetic signal/noise ratio (Zardoya and Meyer, 2001) in the first sequence data set.

All phylogenetic analyses performed in this study recovered the basommatophoran pulmonate *Siphonaria* within Opisthobranchia (Grande et al., 2004). Definition of Opisthobranchia is currently vague because of the retention of primitive gastropods characters in the least derived members of Opisthobranchia (Fretter and Graham, 1949, 1962; Ghiselin, 1966; Gosliner, 1981; Haszprunar and Huber, 1990; Köhler, 1893; Mikkelsen, 2002; Rigby, 1965; Robertson, 1973; Salvini-Plawen,

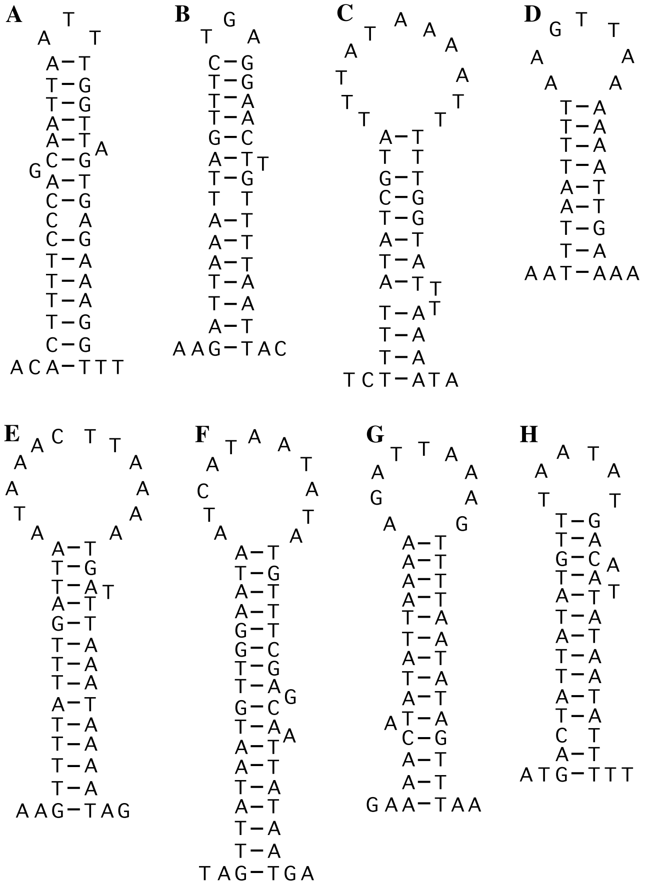

Fig. 3. Examples of putative helical–loop structures within Domain III of the mitochondrial *rrnL* gene in Nudipleura. These structures are absent in the other taxa studied. (A) *Hancockia uncinata*; (B) *Flabellina affinis*; (C) *Eubranchus* sp.; (D) *Bathyberthella antartica*; (E) *Pleurobranchaea meckeli*; (F) *Rostanga pulchra*; (G) *Aldisa banyulensis*; (H) *Triopha maculata*.

1991; Tillier, 1984). Our results prompt for a re-evaluation of the homology and states of the morphological characters of *Siphonaria* taking into account its new phylogenetic position within opisthobranchs as well as for a revision of the morphological synapomorphies that diagnose Opisthobranchia.

The basal position of the sacoglossan *Ascobulla* with respect to the other opisthobranchs was also recovered in all phylogenetic analyses. This result is in agreement with previous morphological studies that have shown *Ascobulla* to be a very primitive taxon within opisthobranchs with plesiomorphic characters such as head shield, external shell, and plicatidium (Jensen, 1996; Mikkelsen, 1996; Schmekel, 1985). Previous studies suggested some members of Architectibranchia (*Acteon* or alternatively *Ringicula*) as the most basal opisthobranchs (Gosliner, 1981; Mikkelsen, 1996, 2002; Schmekel, 1985). To validate this hypothesis, and to search for the origin of the opisthobranchs, *Acteon* and *Ringicula* should be included in future molecular analyses.

All phylogenetic analyses performed in this study recovered two distinct lineages within Opisthobranchia that await formal description: one included Cephalaspidea, Anaspidea, and Tylodinoidea, and the other included Architectibranchia and Nudipleura. Cephalaspidea are characterized by two morphological synapomorphies (Table 1 and Fig. 4): the presence of three gizzard plates and flexed/exogyrous ciliated strips (Mikkelsen, 2002). Our results support their monophyly and the existence of three distinct lineages within this group: Bulloidea, Philinoidea, and Runcinoidea that are represented by *Haminoea*, *Philine* + *Chelidonura*, and *Runcina*, respectively. Of them, only the assignment of Runcinoidea to Cephalaspidea was questioned (Odhner, 1968). However, characters in the nervous and reproductive systems (with open seminal groove and spermatic bulb) support their affinities to cephalaspideans (Kress, 1977; Schmekel, 1985).

Members of the Architectibranchia were traditionally included within Cephalaspidea, although recent morphological studies showed that both groups were only united by plesiomorphies (Mikkelsen, 1996, 2002). Our results supported radically different origins for Cephalaspidea and Architectibranchia. As pointed out by Mikkelsen (2002), Architectibranchia are likely not monophyletic, and more representatives of the group need to be included in future molecular phylogenetic analyses to resolve their systematics.

The monophyly of Anaspidea as recovered by our analyses is well supported both on morphological (Mikkelsen, 1996, 2002; Schmekel, 1985) and molecular grounds (Medina and Walsh, 2000; Thollesson, 1999b). The group is defined by the presence of a filter chamber (Mikkelsen, 2002) (Table 1 and Fig. 4).

Notaspidea are currently divided into Tylodinoidea and Pleurobranchoidea (Rudman and Willan, 1998). However, according to Schmekel (1985), all the characters that defined the Notaspidea were clearly plesiomorphies and Tylodinoidea as well as Pleurobranchoidea were so divergent in their morphology that they did not seem to share the same origin. Our results support Schmekel (1985) views. In our study Tylodinoidea was recovered as the sister group of Cephalaspidea. In fact, several characters such as an open seminal groove and non-retractile penis, an albumen gland, plates in the gizzard and absence of blood gland suggest that Tylodinoidea is more closely related to Anaspidea and Cephalaspidea than to Pleurobranchoidea (Table 1 and Fig. 4). According to our results, Pleurobranchoidea is closely related to Nudibranchia (but see below).

Wägele and Willan (2000) suggested close phylogenetic relationships between Pleurobranchoidea and Nudibranchia, and defined the clade Nudipleura to group them together. Nudipleura was diagnosed by the possession of a blood gland, androdiaulic reproductive system, and the lack of osphradium (Wägele and Willan,

Table 1
Morphological and molecular features that support the recovered clades within the molecular phylogenetic hypothesis

Taxa	Node	Feature
Nudipleura	A	Presence of a helical–loop structure within Domain III of the mitochondrial *rrnL* gene
	B	Absence of the osphradium[a]
Pleuroanthobranchia	C	Presence of blood gland, presence of calcareous spicules in the integument and presence of a caecum with an opening directly into the stomach
Anthobranchia	D	Presence of a caecum lined with ciliated epithelium, a notum overgrowing head and enclosing rhinophores during ontogeny, and postero-median site of anus, nephroproct, and anal gills[a]
Cephalaspidea + Anaspidea + Tylodinoidea	E	Presence of an open seminal groove and non-retractile penis, an albumen gland, plates in the gizzard, and absence of blood gland
Anaspidea	F	Presence of a filter chamber[b]
Cephalaspidea	G	Presence of three gizzard plates and flexed/exogyrous ciliated strips[b]
Philinoidea	H	Presence of an indented rachidian, absence or even loss of gizzard spines, flexed ciliated strips, and carnivorous feeding habits[b]
Architectibranchia	I	Reduction of the stomach and rotation of the mantle cavity opening from anterior to lateral[b]
Cladobranchia	J	Absence of the primary gills, aliform jaws, absence of the bursa copulatrix, and absence of the blood gland[a]
Pleurobranchoidea	K	Presence of midgut acid gland, pedal gland in mature sexual individuals, and the presence of a narrow oral veil in relation to the body width[c]

See Fig. 4 for the identification of the nodes.

[a] Wägele and Willan (2000).

[b] Mikkelsen (2002).

[c] Willan (1987).

2000). However, the possession of a blood gland is not shared by all nudibranchs (Minichev, 1970) and the androdiaulic reproductive system is also present in other architectibranchs and sacoglossans (Ghiselin, 1966; Mikkelsen, 1996). Therefore, the lack of osphradium is the only well-defined synapomorphy of Nudipleura (Table 1 and Fig. 4). In the present study, both phylogenetic analyses of the primary sequence data, and the shared presence of a helical–loop structure within Domain III of the *rrnL* gene in all Pleurobranchoidea and Nudibranchia studied taxa strongly supported the monophyly of Nudipleura. The helical–loop structure within Domain III of the *rrnL* gene was previously reported in basal gastropod lineages whereas it was absent in pulmonates and in the architectibranch *Pupa* (Lydeard et al., 2000, 2002). The new sequences presented in this study show that this helical–loop structure within Domain III of the *rrnL* gene is also present in Nudipleura.

Although the monophyly of Nudipleura has not been questioned, that of Nudibranchia is controversial (Minichev, 1970; Schmekel, 1985; Thollesson, 1999b; Wägele and Willan, 2000; Wollscheid and Wägele, 1999). There are two major lineages within nudibranchs: Anthobranchia and Dexiarchia (Cladobranchia + Doridoxa) (Schrödl et al., 2001; Wägele and Willan, 2000). Some characters such as solid rhinophores, absence (through loss) of the shell, pericardial complex orientated longitudinally and the presence of specialized vacuolated epithelium, were proposed to support the monophyly of Nudibranchia (Wägele and Willan, 2000). However, the absence of the shell is not exclusively restricted to nudibranchs among opisthobranchs (Rudman and Willan, 1998), and the pericardial complex orientated longitudinally is also found in Pleurobranchoidea (Willan, 1987). Minichev (1970) defended different (and in some cases even opposite) evolutionary trends in respiratory, circulatory, and reproductive systems between the two main groups of nudibranchs suggesting different origins for them, and therefore proposed their classification into different orders.

The molecular phylogeny recovered Pleurobranchoidea as sister group of Anthobranchia rendering Nudibranchia paraphyletic. Therefore, we formally introduce the name Pleuroanthobranchia new taxon for the group formed by Pleurobranchoidea and Anthobranchia. The new taxon is defined by the presence of blood gland, calcareous spicules in the integument and a caecum directly opened into the stomach (Table 1 and Fig. 4).

Anthobranchia were recovered monophyletic. This is in agreement with phylogenetic analyses based on morphological data that recognized the following synapomorphies of the group: presence of a caecum lined with ciliated epithelium, a notum overgrowing head and enclosing rhinophores during ontogeny, a postero-median site of anus, presence of a nephroproct, and anal gills (Wägele and Willan, 2000) (Table 1 and Fig. 4). However, our results did not support the traditional subdivision of Anthobranchia into Cryptobranchia and Phanerobranchia based on the presence or absence of a gill pocket, respectively. Neither the phanerobranchs (*Onchidoris*, *Ancula*, *Triopha*, *Tambja*, and *Roboastra*) nor the crytobranchs (*Doris*, *Chromodoris*, *Aldisa*, *Cadlina*, *Discodoris*, *Rostanga*, and *Platydoris*) formed monophyletic groups. Therefore, the protective cavity for the gills in Anthobranchia must have evolved

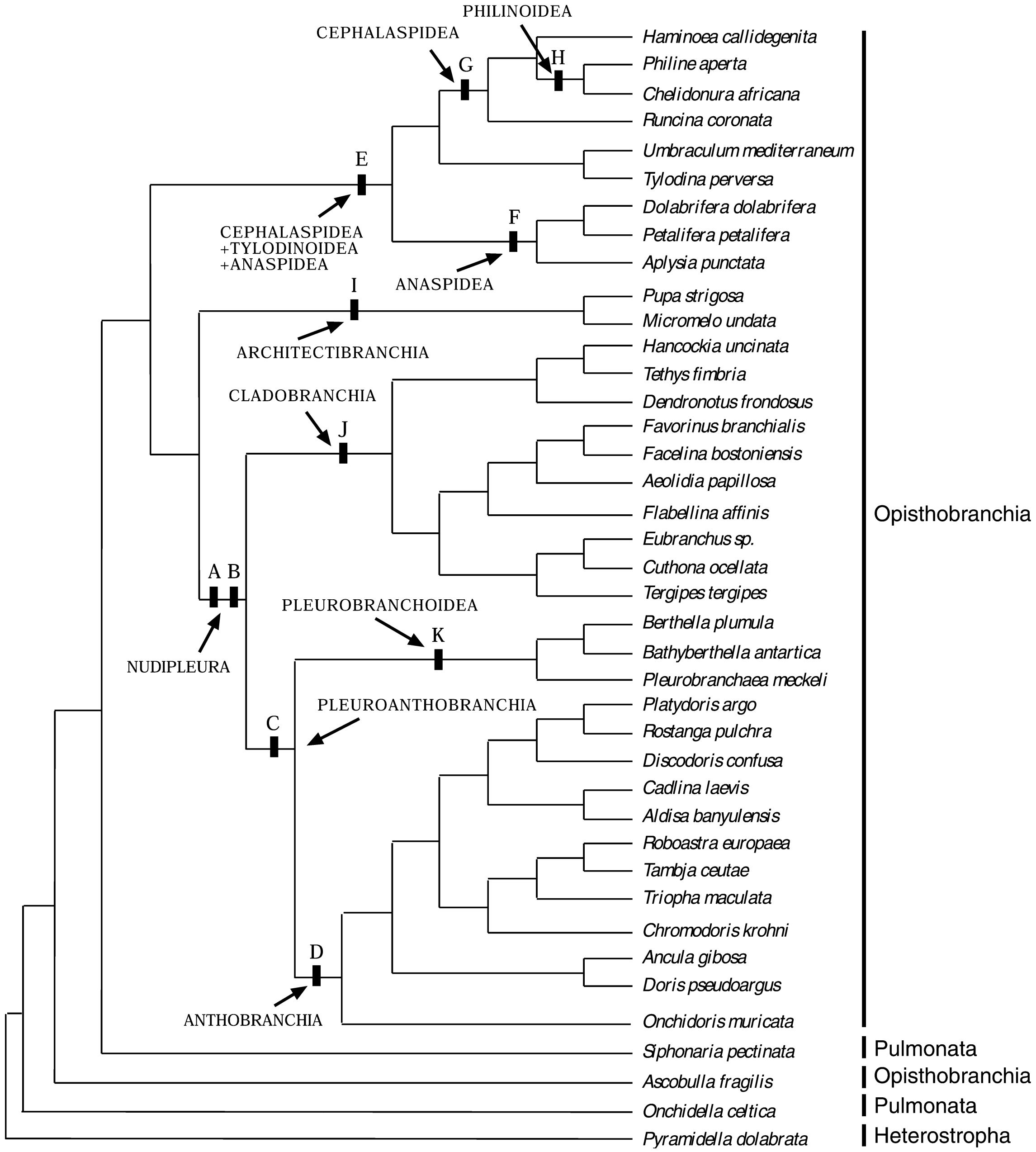

Fig. 4. Review of molecular and morphological features mapped onto the recovered phylogeny. The different clades are identified by their name. Letters indicate molecular and morphological synapomorphies (see Table 1).

several times independently during the evolutionary history of the group.

Cladobranchia were recovered monophyletic in this study although there are no morphological synapomorphies supporting this clade (Wägele and Willan, 2000). Among cladobranchs, the monophyly of Aeolididae was recovered but not those of Dendronotoidea (*Hancockia*, *Tethys*, and *Dendronotus*) and Tergipedidae (*Tergipes* and *Cuthona*).

The molecular phylogeny reconstructed in this study provides new insights on the relationships and evolutionary trends within Opisthobranchia. The basommatophoran *Siphonaria* is recovered within opisthobranchs, and prompts for a more complete analysis of the phylogenetic relationships between opisthobranchs and pulmonates to test the monophyly of each group. Our phylogenetic hypothesis supports, on one side a common ancestor for Anaspidea, Tylodinoidea, and Cephalaspidea (characterized by the presence of an open seminal groove and non-retractile penis, an albumen gland, plates in the gizzard and the absence of blood gland) and, on the other side a common origin for Architectibranchia and Nudipleura (sharing similarities in their circulatory system). Our data support the monophyly of all the studied group within these two main lineages with the exception of Nudibranchia. More representatives of Sacoglossa and Architectibranchia need to be included in future studies to test their monophyletic origins and to corroborate their basal position within opisthobranchs. Further investigations should also involve the remaining orders of Opisthobranchia (Thecosomata, Gymnosomata, Acochlidiomorpha, and Rhodopemorpha) not considered in the present study.

Acknowledgments

G. San Martín, X. Turón, E. Rolán, G. Calado, and M. Schrödl collaborated in the species sampling. C.G. was sponsored by a predoctoral fellowship of the Ministerio de Ciencia y Tecnología. This work received financial support from projects of the Ministerio de Ciencia y Tecnología to J.T. (REN2000-0890/GLO), to J.L.C. (REN2001-1956-C17-02/GLO) and to R. Z. (REN2001-1514/GLO).

Appendix A

List of samples analyzed in this study

Species	Locality	GenBank Accession Nos.
Opisthobranchia		
Architectibranchia		
Pupa strigosa[a]	—	AB028237
Micromelo undata	Cape Verde Islands	AY345014
Cephalaspidea		
Bulloidea		
Haminoea callidegenita	Pontevedra, N Spain	AY345015
Philinoidea		
Philine aperta	Murcia, SE Spain	AY345016
Chelidonura africana	Porto Santo, Madeira Islands	AY345017
Runcinoidea		
Runcina coronata	Sagres, Portugal	AY345018
Anaspidea		
Aplysia punctata	Pontevedra, NW Spain	AY345019
Petalifera petalifera	Murcia, SE Spain	AY345020
Dolabrifera dolabrifera	Cape Verde Islands	AY345021
Sacoglossa		
Ascobulla fragilis	Murcia, SE Spain	AY345022
Tylodinoidea		
Umbraculum mediterraneum	Gerona, NE Spain	AY345023
Tylodina perversa	Porto Santo, Madeira Islands	AY345024
Nudipleura		
Pleurobranchoidea		
Berthella plumula	Pontevedra, NW Spain	AY345025
Pleurobranchaea meckeli	Gerona, NE Spain	AY345026
Bathyberthella antartica	Antartica	AY345027
Nudibranchia		
Cladobranchia		
Aeolidia papillosa	Pontevedra, NW Spain	AY345028
Facelina bostoniensis	Clachan Seil, Scotland	AY345031
Tergipes tergipes	Clachan Seil, Scotland	AY345032
Flabellina affinis	Murcia, SE Spain	AY345055
Tethys fimbria	Tarragona, NE Spain	AY345035
Dendronotus frondosus	Oban, Scotland	AY345041
Cuthona ocellata	Sagres, Portugal	AY345043
Favorinus branchialis	Oban, Scotland	AY345042
Eubranchus sp.	Sagres, Portugal	AY345046
Hancockia uncinata	Sines, Portugal	AY345047

Appendix A (*continued*)

Species	Locality	GenBank Accession Nos.
Anthobranchia		
Roboastra europaea[a]	—	AY083457
Ancula gibbosa	Kingsbarns, Scotland	AY345029
Doris pseudoargus	Kingsbarns, Scotland	AY345030
Onchidoris muricata	Clachan Seil, Scotland	AY345033
Cadlina laevis	Kinkell Braes, Scotland	AY345034
Chromodoris krohni	Murcia, SE Spain	AY345036
Platydoris argo	Ceuta, Strait of Gibraltar	AY345037
Tambja ceutae	Porto Santo, Madeira Islands	AY345038
Aldisa banyulensis	Porto Santo, Madeira Islands	AY345039
Discodoris confusa	Porto Santo, Madeira Islands	AY345040
Rostanga pulchra	California, West of USA	AY345044
Triopha maculata	California, West of USA	AY345045
Pulmonata		
Systelommatophora		
Onchidella celtica	Ceuta, Strait of Gibraltar	AY345048
Basommatophora		
Siphonaria pectinata	Ceuta, Strait of Gibraltar	AY345049
Other Gastropoda		
Pyramidelloidea		
Pyramidella dolabrata	Annobon Island, Gulf of Guinea	AY345054

[a] Directly retrieved from GenBank.

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