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Performance of 18S rRNA in Littorinid Phylogeny (Gastropoda: Caenogastropoda)

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Abstract. In the past, 18S rRNA sequences have proved to be very useful for tracing ancient divergences but were rarely used for resolving more recent ones. Moreover, it was suggested that the molecule does not contain useful information to resolve divergences which took place during less than 40 Myr. The present paper takes littorinid phylogeny as a case study to reevaluate the utility of the molecule for resolving recent divergences. Two data sets for nine species of the snail family Littorinidae were analyzed, both separately and combined. One data set comprised 7 new complete 18S rRNA sequences aligned with 2 published littorinid sequences; the other comprised 12 morphological, 1 biochemical, and 2 18S rRNA secondary structure characters. On the basis of its ability to confirm generally accepted relationships and the congruence of results derived from the different data sets, it is concluded that 18S rRNA sequences do contain information to resolve ''rapid'' cladogenetic events, provided that they occurred in the not too distant past. 18S rRNA sequences yielded support for (1) the branching order (*L. littorea*, (*L. obtusata*, (*L. saxatilis*, *L. compressa*))) and (2) the basal position of *L. striata* in the *Littorina* clade.

Key words: 18S rRNA — Littorinidae — Molecular phylogeny — Morphological phylogeny — *Littorina* — *Littoraria* — *Nodilittorina* — *Melarhaphe*

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Introduction

The 18S rRNA molecule is a popular phylogenetic marker for tracing relationships among distantly related taxa (e.g., Woese and Fox 1977; Cedergen et al. 1988; Gouy and Li 1989; Hasegawa et al. 1993). Yet 18S rRNA data often seem to fail when used for inferring relationships among spiralian phyla, which are supposed to have radiated in a period of some tens of millions of years or less during the so-called Cambrian radiation (e.g., Bergström 1991; Valentine 1991; Adoutte and Philippe 1993; Bowring et al. 1993; Graham et al. 1995; Winnepenninckx et al. 1995a, 1996; Mackey et al. 1996). This issue was studied by Philippe et al. (1994), who concluded that the currently available 18S rRNA sequences cannot unambiguously resolve cladogenetic events separated by less than 40 Myr. Earlier, Hillis and Dixon (1991) had postulated that 18S rRNA sequences are unsuitable for comparing taxa that diverged since the Cretaceous. Hence, 18S rRNA data are not expected to be reliable for tracing relationships among recently diverged taxa, such as closely related species and genera which are of a more recent origin and which may have diverged in less than 40 Myr. Nevertheless, 18S rRNA sequences have been used with fairly good results in phylogenetic analyses of congeneric species (e.g., Bernardi and Powers 1992; Riutort et al. 1992; Baur et al. 1993; Johnston et al. 1993; Lumb et al. 1993; Rice 1993; Han and McPheron 1994; Wright and Lynn 1995; Harasewych et al. 1997; Kaukas and Rollinson 1997). Thus it may be questioned whether the minimal divergence time needed for 18S rRNA to become a reliable phylo-

Table 1. Sampling sites, DNA sources, and 18S rRNA data of littorinid species used in this study

Species	Locality	DNA source	Chain length	% GC content	EBI accession no.
Littorina striata[a]	Faial (Azores)	Digestive gland	1831	50.49	Y11750
Littorina compressa[a]	Roscoff (France)	Digestive gland	1826	50.55	Y11752
Littorina saxatilis[a]	Trondheim (Norway)	Digestive gland	1826	50.47	Y11751
Littorina obtusata	Oosterschelde (Netherlands)	Albumin gland + muscle tissue	1823	50.61	Y94274
Littorina littorea	Oostende (Belgium)	Foot + digestive gland	1834	50.66	Y91970
Nodilittorina punctata M[a]	Brucoli (Italy)	Soft body part[b]	1835	50.30	Y11753
Nodilittorina punctata A[a]	Cape Verde Islands	Soft body part[b]	1835	50.35	Y11755
Littoraria undulata[a]	Laing Island (Papua New Guinea)	Soft body part[b]	1833	50.14	Y11756
Melarhaphe neritoides[a]	Malta	Soft body part[b]	1831	50.41	Y11754

[a] Species for which the 18S rRNA sequence was determined in this study.
[b] The gastrointestinal system was removed prior to DNA extraction.

genetic marker is indeed invariant with respect to the age of the clades involved or whether it may be shorter in younger radiations.

Here we investigate this issue by assessing the utility of 18S rRNA to resolve phylogenetic relationships among periwinkles of the genus *Littorina.* Species of this genus are common on Atlantic rocky shores, where they have been studied intensively. As their phylogenetic relationships and divergence times, which range between approximately 40 and less than 2 Myr ago, are reasonably well-known, they provide a good model to assess the resolving power of 18S rRNA sequences in clades, whose divergence times are shorter than the limit proposed by Philippe et al. (1994).

In this context, we test whether 18S rRNA sequences confirm the association of *L. striata* with the genus *Littorina* and support the branching order (*L. littorea* (*L. obtusata* (*L. compressa, L. saxatilis*))). The latter result is generally accepted on the basis of morphological data (Reid 1990, 1996; Reid et al. 1996), allozyme analyses (Ward 1990; Backeljau and Warmoes 1992; Zaslavskaya et al. 1992; Zaslavskaya 1995), and mtDNA sequences (Reid et al. 1996) and involves divergence times ranging between approximately 1 and 10 Myr (Reid 1996). *L. striata,* on the other hand, has previously been associated with *Melarhaphe* (e.g., Fisher 1967; Nordsieck 1982) or *Nodilittorina* (e.g., Rosewater 1970, 1981; Bandel and Kadolsky 1982). Yet recent morphological (Reid 1989, 1990, 1996), allozyme (Backeljau and Warmoes 1992), and mtDNA analyses (Rumbak et al. 1994; Reid et al. 1996) suggested that the species represents the most basal branch in the genus *Littorina.* Nevertheless, the evidence for this relationship is still somewhat ambiguous, as it is based on (1) only two morphological synapomorphies, *viz.,* the absence of rod-pieces in the paraspermatozoa and the presence of two consecutive loops of the spiral egg groove in the pallial oviduct (Reid 1996); (2) data for nine allozyme loci, of which the distance matrix analysis placed *L. striata* with *Littorina* only with the distance Wagner algorithm, and not with any other tree building method (Backeljau and Warmoes 1992; Backeljau et al. 1994); and (3) a mtDNA study in which the basal position of *L. striata* within *Littorina* was supported by 12S rRNA data but contradicted by 16S rRNA data (Reid et al. 1996). Since *L. striata* diverged roughly 40 Myr ago [(Reid 1996); 32.47–43.93 Myr ago according to Reid et al. (1996)], one would expect that this cladogenetic event might be near the resolution limit of 18S rRNA data.

We determined seven complete littorinid 18S rRNA sequences and produced a parallel ''traditional'' data matrix based on morphological characters and features derived from the 18S rRNA secondary structure. We then explored the relative performance of the molecular and ''traditional'' data by a separate and combined analysis of both data sets. We also assessed the strength of 18S rRNA data in resolving littorinid phylogeny compared to previously published mitochondrial 12S rRNA, 16S rRNA, and cytochrome *b* sequences (Reid et al. 1996). Throughout this paper, we use the abbreviation ''*L.*'' for *Littorina,* while the name *Littoraria* is spelled out.

Materials and Methods

DNA Isolation

Collection sites of the species and tissues used for DNA extraction are given in Table 1. DNA was extracted from the digestive gland using the method of Winnepenninckx et al. (1993) or from the complete soft body, by slightly modifying this protocol. Instead of grinding tissues with a pestle and mortar under liquid N_2, they were minced and incubated in CTAB buffer (Winnepenninckx et al. 1993).

Sequence Analysis

The 18S rRNA gene coding region was amplified via PCR using a primer at the 5′ terminus (5′ CTGGTTGATYCTGCCAGT 3′) and one near the 3′ terminus (5′ CYGCAGGTTCACCTACRG 3′) of the gene

Table 2. Dissimilarities (%) among littorinid 18S rRNA sequences[a]

	Lli	*Lob*	*Lco*	*Lsa*	*Lst*	*Lun*	*Np*A	*Np*M	*Mne*
Lob	1.158	0.000							
Lco	1.267	0.551	0.000						
Lsa	1.215	0.442	0.220	0.000					
Lst	1.923	1.982	2.308	2.094	0.000				
Lun	2.974	2.980	3.247	3.035	1.980	0.000			
*Np*A	2.519	2.580	2.849	2.637	1.697	1.812	0.000		
*Np*M	2.411	2.471	2.795	2.582	1.752	1.867	0.218	0.000	
Mne	2.852	3.079	3.183	3.080	2.301	3.024	2.023	2.024	0.000

[a] *Lli, L. littorea; Lob, L. obtusata; Lco, L. compressa; Lsa, L. saxatilis; Lst, L. striata; Lun, Littoraria undulata; Np*A, Atlantic *N. punctata; Np*M, Mediterranean *N. punctata; Mne, Melarhaphe neritoides.*

(Winnepenninckx et al. 1994). Cloning of the PCR fragments was done as described by Winnepenninckx et al. (1995a). Sequences of both strands of the gene were determined using the A.L.F.-express sequencer (Pharmacia, Uppsala, Sweden). Sequencing reactions were performed with the Thermo sequenase fluorescent labeled primer cycle sequencing kit (Amersham, Buckinghamshire, UK) using the primers of Winnepenninckx et al. (1994) and two universal M13 primers supplied with the kit.

Data Analysis

New 18S rRNA sequences were aligned with the complete 18S rRNA sequences of *L. littorea* and *L. obtusata* (Winnepenninckx et al. 1996, 1998). Aligning was done by hand, taking into account secondary structure features. The alignment may be obtained upon request. For the comparison with other molecular markers, we relied on the 12S rRNA, 16S rRNA, and cytochrome *b* alignments of Reid et al. (1996). Alignments were analyzed using (1) neighbor joining (NJ) (Saitou and Nei 1987), (2) maximum parsimony (MP), and (3) maximum likelihood (ML). NJ analyses of Jukes and Cantor (1969) and Kimura (1980) two-parameter distances were performed with the computer program TREECON (Van de Peer and De Wachter 1993). MP analyses were performed on the parsimony informative sites only using the exhaustive search option of PAUP, Version 3.1.1 (Swofford 1993). Searches were conducted 100 times with multiple random addition of sequences. If more than one MP tree was obtained, data were submitted to the reweight option of PAUP, Version 3.1.1 (Swofford 1993), assigning weights proportional to the maximum rescaled consistency index over all trees in memory. ML trees were calculated using either FastDNAML, Version 1.0 (Olsen et al. 1994), with the global rearrangement and jumble option invoked, or PUZZLE, Version 2.5.1 (Strimmer and von Haeseler 1996), which searches trees via the quartet puzzling algorithm.

The morphological data set of Reid (1996) was updated and extended with data for the non-*Littorina* species (see Tables 4 and 5). In addition, data on radular myoglobin profiles obtained by isoelectric focusing (IEF) (Medeiros et al. 1998) and features of the secondary structure of the 18S rRNA molecule were added to the morphological data set. We did not consider previously published allozyme data because such data are lacking for *Littoraria undulata* and *N. punctata* from the Cape Verde Islands. Moreover, recording allozyme data for parsimony analysis remains a controversial issue (e.g., Backeljau and Warmoes 1992; Murphy 1993). MP analyses on the morphological or combined data set were performed with the exhaustive search option of PAUP, Version 3.1.1.

The stability of all NJ, MP, and ML trees was tested by bootstrapping over 1000 replicates. Only bootstrap values higher than 70% were considered significant (Hillis and Huelsenbeck 1992). In addition, decay indices (Bremer 1988; Donoghue et al. 1992) were calculated for the MP trees. The quartet puzzling tree search generates support values for each internal branch, which have the same practical meaning as bootstrap values (Strimmer and von Haeseler 1996). The strength of the signal in the different data sets was assessed by the tree-length distribution test of Hillis (1991), via the exhaustive search option of PAUP, Version 3.1.1.

Secondary structure models of littorinid 18S rRNA sequences were constructed on the basis of the model of Van de Peer et al. (1998) and drawn using a program developed by Winnepenninckx et al. (1995b).

Results

18S rRNA Sequence Alignment, Information Content, and Secondary Structure

The length, GC content, and EBI accession numbers of the littorinid 18S rRNA sequences used in this study are given in Table 1. There is no base composition bias that may compromise phylogenetic inference. The percentage interspecific dissimilarities (Table 2) between *Littorina* are higher than corresponding values calculated for other molluscan genera, such as for the bivalve genus *Spisula* (mean dissimilarity among *S. solida, S. solidissima,* and *S. subtruncata* is 0.580%) or *Mytilus* (mean dissimilarity among *M. californianus, M. edulis, M. galloprovincialis,* and *M. trossulus* is 0.654%). Our alignment included two 18S rRNA sequences from *N. punctata,* one derived from an Atlantic and one from a Mediterranean specimen (Table 1). Throughout this paper, the two specimens are indicated as *N. punctata* A and *N. punctata* M, respectively. Interestingly, the sequence divergence between the Atlantic and the Mediterranean *N. punctata* (Table 2) is of the same magnitude as between the species *L. saxatilis* and *L. compressa.* Yet it is too early to expand on the possible taxonomic and/or phylogeographic implications of this observation.

According to Hillis (1991), data sets containing phylogenetic signal result in significantly left-skewed distributions of tree lengths; those containing random noise result in symmetrical distributions. Our 18S rRNA alignment includes 56 parsimony informative sites and the distribution in Fig. 1 has a g_1 value of −0.76, which is considerably less than the 99% critical value (between

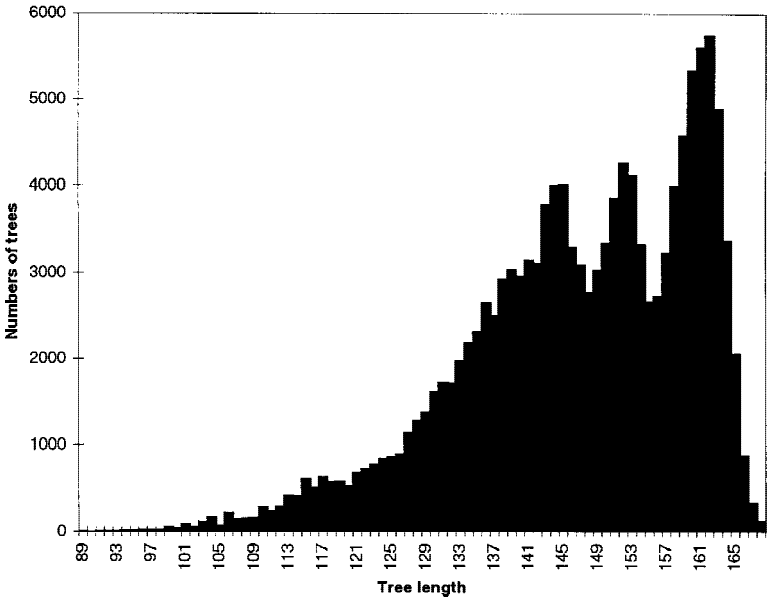

Fig. 1. Frequency distribution of tree lengths on the basis of an alignment of nine complete 18S rRNA sequences.

−0.43 and −0.44 for nine taxa and 50–100 parsimony informative characters) given by Hillis and Huelsenbeck (1992) for structured data sets. This suggests that our 18S rRNA data set contains significant phylogenetic signal.

Many differences between the 18S rRNA sequences are found in the V2 region (Neefs et al. 1990) of the molecule, provoking differences in the secondary structure of this region (Fig. 2), e.g., in the length of helix 10 (7–9 base pairs; bp) and in the structure of the terminal part of helix E10-1, which reveals four states: (1) the middle bulge is asymmetrical (four nucleotides on one site and six on the other) and there is a 3-bp terminal helix part (Figs. 2G–I), (2) the middle bulge is asymmetrical (three and four nucleotides) and there is a 3-bp terminal helix part (Figs. 2B, D, and E), (3) the middle bulge is asymmetrical (six and eight nucleotides) and the terminal helix consists of 5 bp (Figs. 2C and F), and (4) the middle bulge is asymmetrical (two and five nucleotides) and the terminal helix consists of 6 bp (Fig. 2A).

Molecular Phylogenetic Analyses

Analyses of the nine littorinid 18S rRNA sequences with different prosobranchs as outgroup (results not shown) confirmed previous studies suggesting that *Melarhaphe* is the most basal branch within the Littorinidae (e.g., Reid 1989; Backeljau and Warmoes 1992). Therefore, subsequent analyses were performed with *M. neritoides* as outgroup. The NJ tree of Jukes and Cantor (1969) and Kimura (1980) distances (Fig. 3A) shows that (1) *L. obtusata* is a sister group to the cluster of *L. saxatilis* and *L. compressa* (the three species belong to the subgenus *Neritrema,* (2) *L. littorea* is a sister taxon to *Neritrema,* and (3) *Littoraria* and *Nodilittorina* appear to be sister taxa. Although not supported by bootstrap values, *L. striata* is included in the *Littorina* cluster. The MP analysis yields three equally parsimonious trees (length = 89 steps), of which the consensus tree (Fig. 3B) confirms the NJ results except for the joining of *Nodilittorina* and *Littoraria.* However, successive weighting produces a single MP tree (Fig. 3C), which confirms all findings of the NJ analysis (Fig. 3A). The same results are also found by the two ML methods implemented by the computer programs FastDNAML and PUZZLE (Fig. 3C). Both the successive weighting MP analysis and the ML analyses yield significant bootstrap support for the status of *L. striata* as the most basal branch within the *Littorina* clade (Fig. 3C). The existence of a *Littoraria–Nodilittorina* clade is significantly supported only by the quartet puzzling ML method. The fact that the reliability value obtained by the quartet puzzling method is much higher than the bootstrap values obtained by successive weighting and the ML method of FastDNAML may be a nice illustration of the recent findings of Cao et al. (1998). They stated that some of the reliability values of the quartet puzzling method may be misleadingly high relative to the bootstrap probabilities.

Finally, the performance of 18S rRNA in retracing the generally accepted topology (((*L. saxatilis, L. compressa*), *L. obtusata*), *L. littorea*) was assessed by comparing the NJ, MP, and ML trees of the 18S rRNA, 12S rRNA, 16S rRNA, and cytochrome *b* sequence data of these five *Littorina* species (trees not shown). *L. striata* is used as outgroup. Table 3 lists the bootstrap values obtained for the different nodes in the five-species trees. Only the 18S rRNA and cytochrome *b* trees consistently confirmed the generally accepted *Littorina* topology with significant bootstrap support (Table 3). The g_1 value of

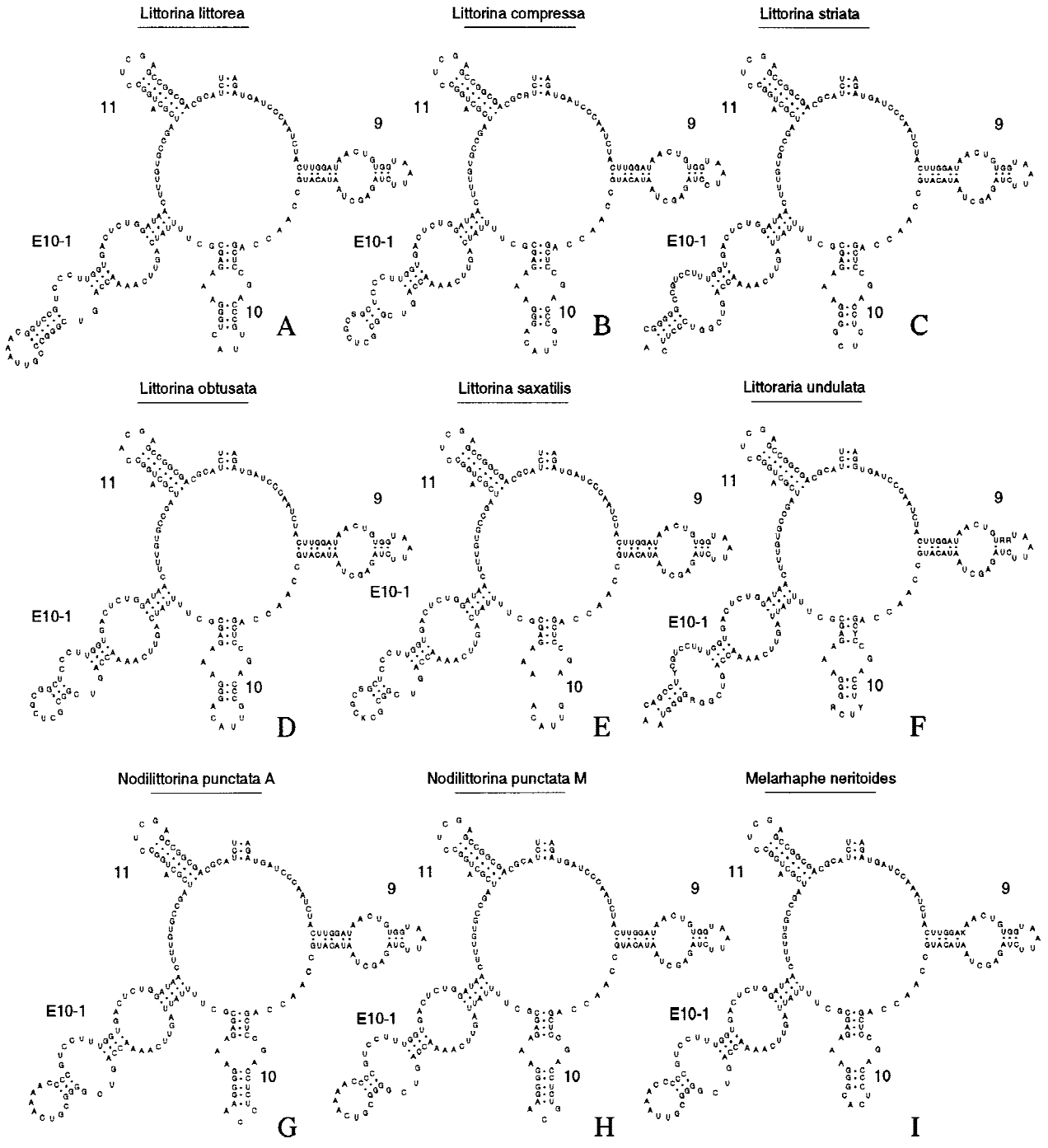

Fig. 2. Secondary structure of the V2 region of the littorinid 18S rRNA molecule. Helix numbering is as described by Van de Peer et al. (1998).

the 12S rRNA data set is positive and therefore its tree length distribution is not skewed to the left, indicating that there is no significant structure in this data set. According to the g_1 values of the 16S rRNA and the 18S rRNA data sets, they should be more structured than random data, but only at the 95% significance level [critical value = −0.95 to −0.78 for 10–50 characters (Hillis and Huelsenbeck 1992)]. Only the g_1 value of the cytochrome *b* sequences is lower than the 99% critical value (−0.88 for 50 informative sites).

Morphological Phylogenetic Analyses

A list of the morphological characters and their character states in the different species is given in Tables 4 and 5, respectively. Whenever possible, species-specific character states are used. Otherwise, the ''generalized'' state of the closest supraspecific taxon (i.e., subgenus or genus) is applied. In addition to morphological characters, two characters of the secondary structure of the 18S rRNA molecule (length of helix 10 and structure of the

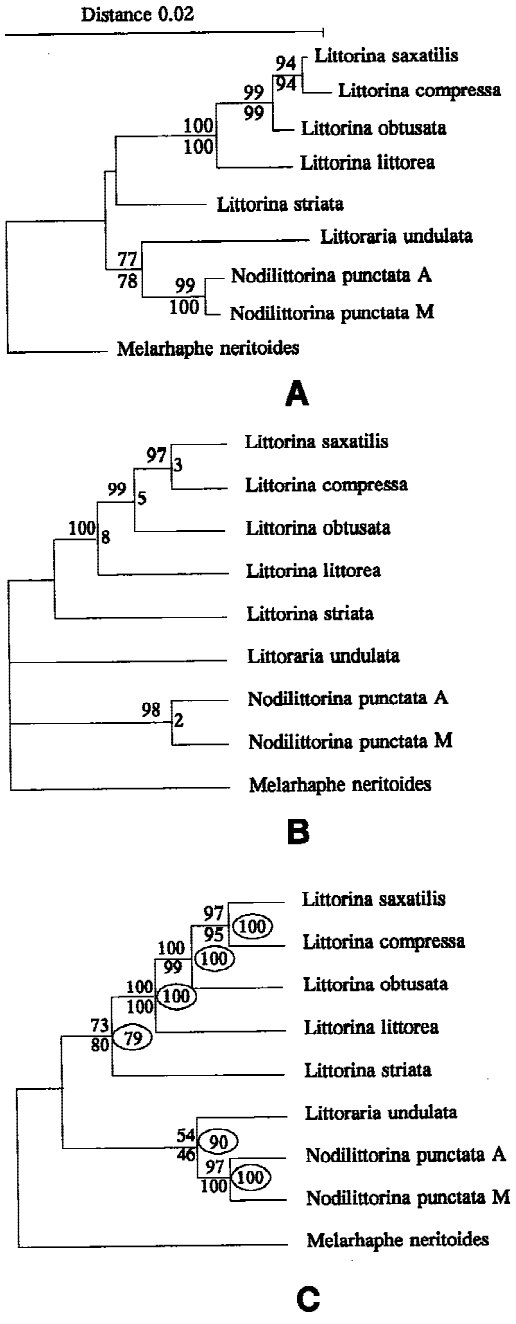

Fig. 3. Littorinid phylogeny on the basis of an alignment of complete 18S rRNA sequences from eight littorinids and *M. neritoides* as the outgroup. **A** NJ tree with percentage bootstrap values of Jukes and Cantor (1969) distances (*above branching points*) and Kimura (1980) distances (*below*.) Only support values higher than 50% are indicated. **B** Consensus tree of the three MP trees (length = 89 steps, CI = 0.787, RC = 0.634) based on 56 informative sites. Figures *above branching points* are percentage bootstrap values (>50%); those to the *right* of them are decay indices. **C** Tree obtained with the ML method of FastDNAML (percentage bootstrap values *below branching points*), with the ML quartet puzzling method (percentage statistical support values *circled*), or by MP analysis with successive reweighting of the data set on the basis of the rescaled consistency indices (percentage bootstrap values *above branching points*).

terminal helix part of E10-1; Fig. 2), as well as a qualitative assessment of IEF radular myoglobin profiles (Medeiros et al., 1998) are included. Figure 4A shows a tree length distribution on the basis of the characters of Table 4. Although no g_1 critical values for five-state character data sets are available (Hillis and Huelsenbeck 1992), it is clear that the distribution is left-skewed.

A MP analysis of the 15 morphological/biochemical characters (Table 4) yields nine equally parsimonious trees (length = 39 steps). Successive weighting has no effect on this number. Their consensus tree does not support the inclusion of *L. striata* in the *Littorina* clade (Fig. 5A), but clusters *L. striata* with the two *N. punctata* specimens. However, only one additional step is required to restore *Littorina* monophyly. The *L. striata–N. punctata* clade is supported by unambiguously traceable changes in two characters, namely, the radular teeth cusps and the outer marginal teeth. The morphological data confirm the sister-group relationship between *L. littorea* and the monophyletic *Neritrema* clade. However, neither the *L. obtusata–L. compressa–L. saxatilis* nor the *Littoraria–*(*Nodilittorina* + *L. striata*)*–Littorina* branching order can be resolved on the basis of these morphological characters. Excluding the three biochemical characters (characters 13–15 in Table 4) yields the same topology and number of trees (length = 30) as shown in Fig. 5A. To test the effect of using (sub)genus-specific instead of species-specific character states, characters for which no species-specific data are available (characters 2, 5, 8, 12) are excluded. Doing so results in an unresolved polychotomy of *Littoraria–Nodilittorina–L. striata–Littorina* (results not shown).

Visual comparison of the secondary structure features reveals that (1) the structure of the terminal part of helix E10-1 (type 2) supports the monophyly of the *Neritrema* clade, (2) the structure of helix 10 suggests a close relationship between Atlantic and Mediterranean *N. punctata*, and (3) the structure of the terminal part of helix E10-1 of *L. striata* resembles most that of *Littoraria undulata* (type 3), even though not all bases of the terminal part of helix E10-1 of the latter species are paired. Except for the latter finding, these results conform with those obtained on the basis of the primary 18S rRNA sequence data.

Total Evidence Analyses

When combining morphological and molecular data, the 18S rRNA secondary structure features (characters 13 and 14 in Table 4) are excluded to avoid the presence of nonindependent characters. The tree length distribution of the total evidence data set is given in Fig. 4B, and as expected, it is skewed to the left. The total evidence MP analysis yields the same topology as the NJ tree on the basis of 18S rRNA data alone. However, the MP tree (length = 123) (Fig. 5B) is equivalent neither to the MP

Table 3. Number of alignment sites, parsimony informative sites, g_1 values, and bootstrap values (%) for *Littorina* clades in the five-species phylogenetic trees obtained on the basis of four molecules[a]

	12S rRNA	16S rRNA	Cytochrome *b*	18S rRNA
Alignment sites	374	444	651	1841
Parsimony-informative sites	9	8	52	11
Parsimony-informative sites/100 sites	2.4	1.8	8.0	0.6
g_1 value	0.126	−1.35	−098	−0.96
Bootstrap support for				
Lco + Lsa	73/na/na	na/na/na	100/100/100	94/98/96
Lco + Lsa + Lob	60/56/na	100/100/1000	100/100/99	97/93/96

[a] *L. striata* was used as outgroup. Bootstrap values are listed for Jukes and Cantor distance analysis, MP, and Fast DNAML analyses, respectively. Abbreviations of species names are as listed in Table 2, footnote a. na, this clade is absent.

trees of the sequence data alone nor to the trees found on the basis of morphological/biochemical data alone. For *L. striata*, a basal position within the *Littorina* clade is suggested, but without significant bootstrap support. High bootstrap support is obtained for the relationships among the *Neritrema* species.

The combined analysis indicates for which part of the tree the information content of a particular data set is weak. The branching pattern of the subgenus *Neritrema*, which remained a trichotomy on the basis of morphological/biochemical data alone, is well resolved by combining the data, pointing to the contribution of the molecular characters in the resolution of this part of the tree. The *Littoraria–Nodilittorina–Littorina* relationship could not be resolved by our MP analysis of 18S rRNA data, while the combined data suggested *Littoraria–Nodilittorina* monophyly, but without significant bootstrap support. When characters for which genus-specific states are used (characters 2, 5, 8, 12) are excluded, the contribution of the morphological characters is apparently insufficient to resolve the *Littoraria–Nodilittorina–Littorina* relationship and the same topology as on the basis of 18S rRNA data alone (Fig. 3B) is obtained.

To reduce the discrepancy in character number between the molecular (56 informative sites) and the morphological/biochemical (13 informative characters) data set, morphological/biochemical characters were given weight 56 and molecular data weight 13. When doing so, a single MP tree was found that confirmed the relationships in the *Neritrema–L. littorea* clade but that placed *L. striata* in the *Nodilittorina–Littoraria* clade as a sister group of the two *Nodilittorina* (not shown).

Discussion

The present results suggest that 18S rRNA sequences can successfully resolve divergences between congeneric species. Indeed, the broadly accepted branching pattern of the species *L. littorea, L. obtusata, L. compressa,* and *L. saxatilis,* which radiated in less than 15 Myr and originated in the Middle Miocene (Reid 1996), could be retraced with high confidence using 18S rRNA sequences. The 18S rRNA and morphological analyses yield congruent results on this point. In this respect, 18S rRNA performed as well as cytochrome *b* and even better than 12S and 16S rRNA, which are often used to study recent phylogenetic relationships (e.g., Milinkovitch et al. 1993; Frye and Hedges 1995). However, the 18S rRNA results for the basal branching order of the genera *Littorina, Nodilittorina,* and *Littoraria* and the position of *L. striata* are less stable. These findings suggest that 18S rRNA sequences may be well suited to trace rapid radiations that took place in less than 40 Myr, on the condition that they occurred ''recently.'' During rapid radiations, only fast-evolving sites will accumulate substitutions that mark the branching order. If such a radiation occurred recently, this information will still be present in the molecule. Yet if a long period has passed since the radiation, the fast-evolving sites containing information on the cladogenetic event will have accumulated additional mutations and the original information will be obscured.

A disadvantage of 18S rRNA (±1800 bp) is the high sequencing effort required to obtain a relatively small number of parsimony informative sites (Table 3). Yet once a few complete sequences are determined, it may be possible to determine the most informative regions of the molecule and to restrict the sequencing work to these. For the present 18S rRNA data set, the majority of informative sites (33 of 56) are found between positions 189 and 252, 668 and 750, and 1729 and 1749 of our alignment, i.e., a stretch of 165 bp.

Although the secondary structure features of the 18S rRNA molecule do not affect the topology of the MP trees, they seem to yield useful clues as to littorinid relationships, except perhaps for the similarity between the secondary 18S rRNA structures of *L. striata* and *Littoraria undulata.* Yet this similarity may be questionable, as all bases of the terminal E10-1 helix part of *L. striata* are paired, whereas this is not the case for *Littoraria undulata,* in which one of them is not.

Furthermore, it seems that different data sets may be informative for different areas of the tree. Yet the combination of morphological and molecular data (e.g., Miyamoto 1985; Kluge 1989; Barrett et al. 1991; Donoghue

Table 4. Characters used in the morphological/biochemical phylogenetic analysis

Character No.	Character	Character state	Description of character state
1	Shell shape	0	*Melarhaphe*-like
		1	*Littoraria*-like
		2	*Nodilittorina*-like
		3	*L. littorea*-like
		4	*L. obtusata*-like
		5	*L. saxatilis*-like
2	Shell mineralogy	0	Aragonite only
		1	Aragonite with outer calcite layer
3	Mamilliform penial glands	0	Absent
		1	1 gland
		2	3 or more glands
4	Simple penial glands	0	Absent
		1	Scattered around penial vas deferens
		2	Forming a discrete glandular disk
5	Paraspermatozoa	0	Rod pieces absent
		1	Rod pieces present
6	Coiling of egg grove of pallial oviduct	0	*Nodilittorina*-like
		1	*L. striata*-like
		2	*L. littorea*-like
		3	*L. obtusata*-like
		4	*L. saxatilis*-like
		5	*Littoraria*-like (single multiple spiral)
7	Jelly gland of pallial oviduct	0	Thin-walled
		1	Enlarged, with thick glandular septa
		2	Modified as brood pouch
8	Egg capsules	0	Pill box
		1	Biconvex disk
		2	Cupola sculptured with two concentric rings
		3	Ovoid, within benthic gelatinous mass
		4	Retained in brood pouch; tin egg covering only
9	Development	0	Planktotrophic
		1	Nonplanktotrophic
		2	Ovoviviparous
10	Cusps of 5 central radular teeth	0	Long, rectangular or pointed
		1	Short, pointed
		2	Short, blunt
11	Outer marginal teeth	0	Outer basal projection, narrow neck
		1	Basal projection indistinct, neck not narrowed
12	Size of salivary glands	0	Small
		1	Enlarged
13	Structure of helix 10	1	7 bp
		2	8 bp
		3	9 bp
14	Structure of helix E10-1	1	Second bulge asymmetric (4–6 nt), Third helix part 3 bp
		2	Second bulge asymmetric (3–4 nt), Third helix part 3 bp
		3	Second bulge asymmetric (6–8 nt), Third helix part 5 bp
		4	Second bulge asymmetric (2–5 nt), Third helix part 6 bp
15	Radular IEF myoglobin profiles	0	*Melarhaphe*-like
		1	*Littoraria*-like
		2	*L. littorea*-like
		3	*L. obtusata*-like

and Sanderson 1994; Jones et al. 1993) remains controversial (e.g., Swofford 1991; de Queiroz 1993; de Queiroz et al. 1995). One of the problems of our total evidence analysis is that the molecular characters outnumber the morphological ones, although the 13 morphological/biochemical characters represent more of the genome than the 56 sequence characters. Differential weighting of the morphological characters shifts *L. striata* from the *Littorina* cluster to the *Littoraria–Nodilittorina* cluster. Hence the size of the different data sets may indeed be relevant for this study. Unfortunately, our weighting scheme is speculative, as one does not know the ''real weight'' of a morphological or biochemical character in comparison to a single nucleotide site. Another problem of our total evidence analysis is the lack of species data for some morphological characters.

Table 5. Data matrix used in the morphological/biochemical phylogenetic analyses (characters listed in Table 4)

	Character no.														
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
M. neritoides	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
Littoraria undulata	1	0[a]	0	2	1	5	0	1	0	1	1	0	1	3	1
N. punctata A	2	0[a]	1	2	1[a]	0	0	2[a]	0	0	0	0[a]	3	1	1
N. punctata M	2	0[a]	1	2	1[a]	0	0	2[a]	0	0	0	0[a]	3	1	1
L. striata	2	0	0	1	0	1	0	2	0	0	0	0	1	3	1
L. littorea	3	1	2	1	0	2	0	1	0	1	1	1	2	4	2
L. obtusata	4	1	2	1	0	3	1	3	1	2	1	1	1	2	3
L. compressa	5	1	2	1	0	3	1	3	1	2	1	1	1	2	2
L. saxatilis	5	1	2	1	0	4	2	4	2	2	1	1	1	2	2

[a] Genus-specific instead of species-specific character state listed.

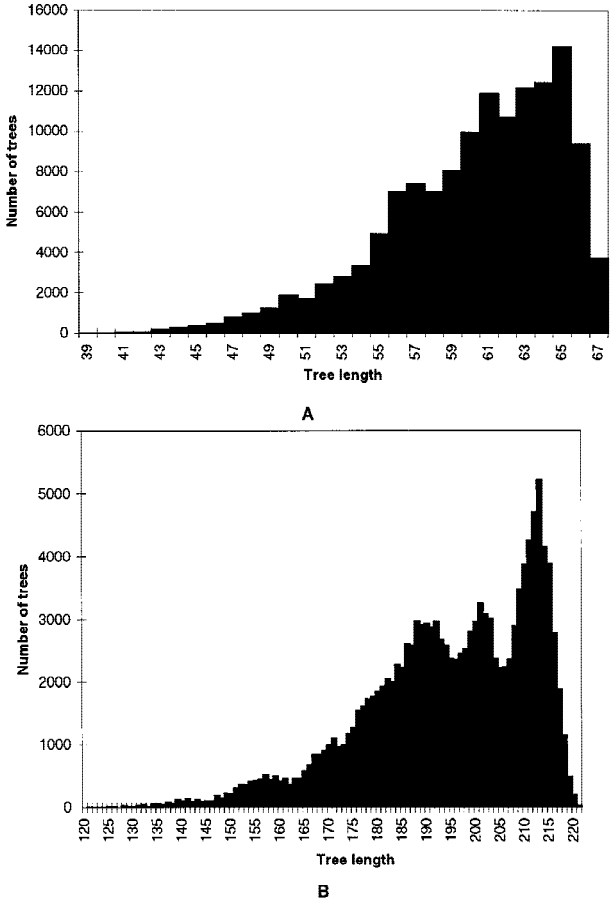

Fig. 4. Frequency distribution of tree lengths on the basis of **A** the morphological/biochemical data matrix in Table 5; **B** a combination of the 18S rRNA sequences with the morphological/biochemical data matrix in Table 5 (excluding characters 13 and 14).

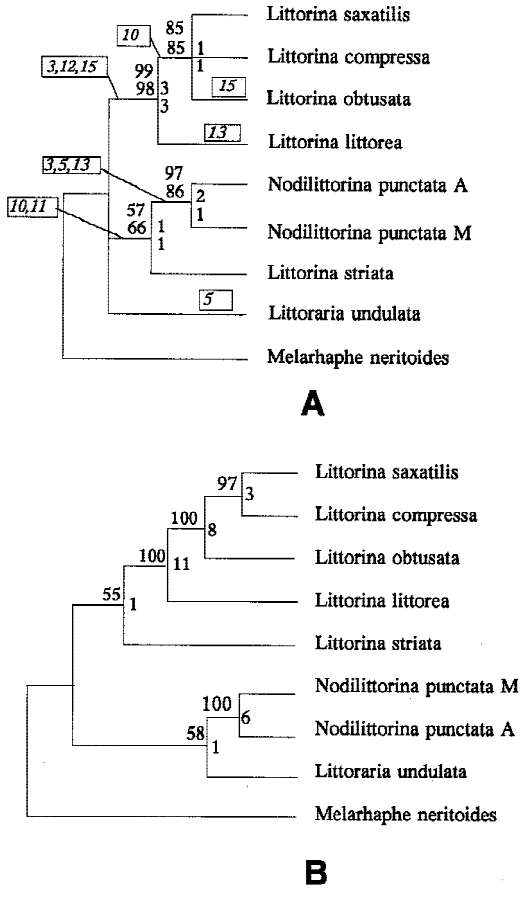

Fig. 5. **A** Consensus tree of the nine MP trees (length = 39, CI = 0.923, RC = 0.834) obtained on the basis of the character matrix listed in Tables 4 and 5. Characters undergoing an unambiguous change at a branch are listed in *boxes*. Figures *above nodes* are percentage bootstrap values (higher than 50%); those to the *right of the nodes* are decay indices. The *upper* bootstrap or decay values are obtained when all characters in Table 5 are analyzed; the *lower* ones indicate values obtained when the morphological characters alone (characters 1–12) are included. **B** MP tree (length = 123, CI = 0.821, RC = 0.677) obtained by combining 18S rRNA sequences with the morphological/biochemical data set in Table 5 (excluding characters 13 and 14). Figures *above branching points* are percentage bootstrap values higher than 50%; those to the *right* of nodes are decay indices.

In this case generalized, higher-level group character states are applied. Sequence data, on the contrary, are species/specimen specific. Hence, total evidence analyses sometimes combine species-specific sequence data with generalized morphological data that do not necessarily apply to the species involved, because the specific character states either are unknown or even differ from

the generalized states. We do not expand on this issue but note that this practice may introduce bias. In the present case, for example, the inclusion of the morphological characters with generalized states provokes a resolution which collapses to an unresolved polychotomy when only the species-specific characters are retained.

The present 18S rRNA data provide new evidence for the basal position of *L. striata* in the *Littorina* clade. This result is independent of the way of analyzing the data and is supported by bootstrapping in the ML and MP successive weighting analyses. The inclusion of *L. striata* in the genus *Littorina* is further supported by mitochondrial sequence data (Rumbak et al. 1994). Although previous morphological analyses already suggested (Reid 1989, 1990, 1996) that *L. striata* belongs to *Littorina*, the current morphological data set is ambiguous on this issue and, in fact, rather places *L. striata* with *Nodilittorina.* This grouping is based on two main features, *viz.*, long radular teeth cusps and outer marginal teeth with an outer basal projection and a narrow neck. If one accepts the basal position of *L. striata* in *Littorina*, then these two characters must be considered as convergences probably due to the similar ecology of *L. striata* and *Nodilittorina* (Reid 1996).

In conclusion, 18S rRNA sequences may be well suited for studying relatively recent cladogenetic events such as the littorinid radiation and may therefore provide a useful marker complementary to mtDNA sequences and traditional data.

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