PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

DNA, Morphology and Fossils: Phylogeny and Evolutionary Rates of the Gastropod Genus Littorina

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DNA, morphology and fossils: phylogeny and evolutionary rates of the gastropod genus *Littorina*

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SUMMARY

Using data from the direct sequencing of fragments of three mitochondrial genes (12S and 16S ribosomal RNA, and cytochrome-*b*; total length 1469 b.p.) we have reconstructed a gene phylogeny for all 19 living species of the gastropod genus *Littorina*. Members of the closely related genera *Nodilittorina*, *Littoraria* and *Mainwaringia* have been used as outgroups, and it appears that *Littorina* is monophyletic. An earlier morphological phylogeny has been revised, and its topology found to be almost entirely consistent with that from the molecular data. The fossil record is sparse, but likewise consistent. A consensus tree is presented, showing clear resolution of basal and terminal branches, and a central unresolved polychotomy. We have used fossil evidence and geological events to estimate the ages of some clades, and thus to calculate average rates of molecular evolution, which in turn provide approximate dates for all branches of the molecular phylogeny. The central polychotomy may be explained by a burst of rapid speciation in the northwestern Pacific during the Middle Miocene, perhaps driven by climatic fluctuation. Our results support the hypothesis that the two clades of *Littorina* in the northern Atlantic originated from Pacific ancestors which took part in the Pliocene trans-Arctic migration of marine organisms.

1. INTRODUCTION

Members of the gastropod genus *Littorina* are abundant and familiar inhabitants of rocky shores throughout the temperate and subarctic regions of the northern hemisphere. As a result of this accessibility, and because of their importance in the community structure of littoral ecosystems (Hawkins *et al*. 1992; Vadas & Elner 1992), all aspects of their biology have been extensively studied (see reviews by Fretter & Graham 1980, 1994; Reid 1996; symposium volumes by Johannesson *et al*. 1990; Grahame *et al*. 1992; Mill & McQuaid 1995). Indeed, *Littorina* is arguably the most thoroughly investigated of all marine gastropod genera. A recent systematic revision of the genus recognizes 19 living species and provides detailed accounts of geographical distributions, life histories and the known fossil record (Reid 1996). With such a wealth of available information, *Littorina* provides an excellent model system for the investigation of macroevolutionary processes in marine invertebrates. In this study we attempt to reconstruct the phylogeny of the entire, apparently monophyletic, radiation of *Littorina* species, using both morphological and molecular datasets, and use it to test two biogeographic hypotheses. No comparable molecular phylogenies of an entire genus of marine gastropods are available, although two molecular studies of smaller, and possibly monophyletic, radiations have been made (*Umbonium* by Ozawa & Okamoto 1993; *Nucella* by Collins *et al*. 1996) and these have produced important insights into the processes of speciation in shallow-water invertebrates of temperate oceans.

Despite numerous studies on the biology and taxonomy of *Littorina*, the macroevolutionary history of the group has been neglected until the last decade. In part, this has been because the fossil record of littorinids is poor, as a consequence of their usual habitat on rocky coasts of moderate to high wave energy, in which conditions are unfavourable for preservation. Furthermore, even if preserved, the shells of the group are rather uniform, with few well-defined characters. As shown by the confused history of the classification of the group, discussed below, shells give only a poor indication of phylogenetic relationships. One early discussion of the evolution of *Littorina* was claimed to have been based on comparison of modern shells and fossils (Golikov & Tzvetkova 1972), but no details were provided to support the proposed evolutionary tree. The limited fossil record has now been examined in detail (Reid 1996), providing important information for this work. The first phylogenetic analyses of *Littorina* were based largely on newly discovered anatomical characters. A preliminary morphological analysis of genera within the family Littorinidae established *Littorina* as a likely monophyletic group (Reid 1986*a*), and this was supported by a more detailed analysis based on representatives of all littorinid subgenera (Reid 1989), which in addition identified *Nodilittorina* as the likely sister-genus. There followed a cladistic analysis of all species then recognized in *Littorina*, using a similar set of morphological characters (Reid 1990*a*).

Molecular techniques now provide us with direct access to the genetic material and make entirely new classes of characters available for phylogenetic re-

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construction. At one step removed from the DNA itself are the gene frequency estimates made possible by allozyme electrophoresis, and the analysis of various measures of genetic distance (Nei 1987). Allozyme studies of this kind have so far been based on sets of only up to eight *Littorina* species, and the results have often been consistent and compatible (Ward 1990; Knight & Ward 1991; Backeljau & Warmoes 1992; Zaslavskaya *et al.* 1992; Zaslavskaya 1995; but see Sundberg *et al.* 1990; Boulding *et al.* 1993; Backeljau *et al.* 1994). So far, only a few studies have employed DNA technology to provide data for phylogenetic analysis. The banding patterns of random-amplified polymorphic DNA (RAPDs) have been used to infer phylogenetic relationships among three sympatric *Littorina* species (Crossland *et al.* 1995). This study is the first to employ DNA sequence data in phylogenetic analyses of *Littorina* species. Preliminary results, based on the 12S ribosomal RNA mitochondrial gene of 11 species of *Littorina*, have already been reported (Rumbak *et al.* 1994), and the full results are presented here.

The main objective of this study was to use mitochondrial DNA sequence data to reconstruct a gene phylogeny of all 19 recognized living species of *Littorina*. With these data it was hoped to test available phylogenies and to obtain a more highly resolved hypothesis of relationships. In particular, two problems were outstanding from previous phylogenetic studies of the group. The analyses of morphology had failed to determine the relationships of the enigmatic genus *Mainwaringia*, with two living species (Reid 1986*b*), which appeared either within the clade of *Littorina* species or in a distant, basal position in the generic-level cladogram (Reid 1989, 1990*a*). The second problem related to the affinities of *Littorina striata*, which consistently appeared as the basal species of the genus in morphological analyses (Reid 1989, 1990*a*), but which in some analyses of allozyme data clustered with other littorinid genera (Backeljau & Warmoes 1992; Backeljau *et al.* 1994), thus questioning the monophyly of *Littorina*. With independent datasets available from morphology, allozymes and DNA sequences, it has been possible to search for congruence among the phylogenetic hypotheses derived from each and to summarize the result as a consensus tree (see review by Miyamoto & Fitch 1995). Before this could be done, however, it was necessary to revise the morphological cladogram of Reid (1990*a*) in the light of recently discovered taxa and the new understanding of certain characters, as described in detail elsewhere (Reid 1996). Information on the fossil record of *Littorina* was taken from a recent monograph (Reid 1996); although the record is too sparse to permit any kind of phylogenetic reconstruction from fossils alone, it has been used here, in combination with geological and distributional data, to estimate the age of certain points on the phylogeny. From these, it has been possible to estimate average rates of molecular divergence, and hence to provide an approximate timescale for the phylogeny. In general, a robust phylogeny is necessary for rigorous testing of macroevolutionary hypotheses. In *Littorina*, a phylogenetic tree has previously been used to support two particular hypotheses of historical biogeography (Reid 1990*b*). One relates to a pattern of vicariant speciation consequent upon range shifts along continental margins that may be induced by climatic change (Valentine & Jablonski 1983; Valentine 1984), predicting a correspondence between latitudinal distribution and phylogenetic position of taxa. The second proposes that *Littorina* took part in the large-scale trans-Arctic interchange of marine organisms that occurred during the Pliocene (review by Vermeij 1991), predicting sister-group relationships between taxa in the northern Pacific and northern Atlantic. These two predictions are tested here, whereas other aspects of the macroevolution of the group, including speciation mechanisms, general biogeographic history and morphological adaptation, are considered elsewhere (Reid 1996).

2. MATERIAL AND METHODS

(*a*) *Systematics, nomenclature, geographical distribution and fossil record of* Littorina *species*

The taxonomy of *Littorina* has undergone extensive revision over the last three decades. As a result of considerable intraspecific variation, traditional characters of the shell are often a poor guide to species identification. With the description of reproductive anatomy, a number of species have been rediscovered or described as new, both in Europe (Sacchi & Rastelli 1966; Heller 1975; Sacchi 1975; Hannaford Ellis 1979) and in the northern Pacific (Habe 1979; Murray 1979; Reid & Golikov 1991; Reid *et al.* 1991). Some of these resolutions of taxonomic problems have been confirmed by genetic studies of allozyme variation (review by Ward 1990). The concept of the genus *Littorina* itself has also undergone revision. Older systems of classification, based mainly on a small set of characters of the shell and radula, included within the genus approximately 50 species, of worldwide distribution (e.g. Rosewater 1970). With the new emphasis on anatomical characters, the definition of the genus was narrowed (Bandel & Kadolsky 1982; Reid 1986*a*), so that it comprised a smaller, apparently monophyletic group, found almost exclusively in the northern hemisphere (Reid 1989, 1990*a*, 1996).

In a review of the family Littorinidae, Reid (1989) listed a total of 18 living species of *Littorina* and two of the enigmatic genus *Mainwaringia*. This same classification was used in the first morphological phylogenetic analysis of *Littorina* (Reid 1990*a*). Since then, there have been a number of taxonomic changes in the genus. These must be briefly reviewed, to explain some of the changes that we make to this earlier analysis. From these earlier morphological studies it was not clear whether the two species of *Mainwaringia* (*M. leithii* (E. A. Smith, 1876) and *M. rhizophila* Reid, 1986) were members of the clade *Littorina*. The molecular data reported here (see Results) show that *Mainwaringia* is not closely related to *Littorina*, and in the revised morphological analysis presented here these two species have therefore been excluded. Of other species accepted by Reid (1990*a*), *L. kurila* (*sensu* Reid, not Middendorff, 1848) has also been excluded, as a

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 1. *List of littorinid species for which mitochondrial gene sequences were obtained, with collection localities and numbers of individuals sequenced*

(List includes 12S sequences already reported by Rumbak *et al.* (1994). A dash indicates that sequence could not be obtained for this species. For authorities of *Littorina* species, see table 4.)

species	locality	12S	16S	cyt-*b*
Littorina striata	El Golfo, Lanzarote, Canary Islands	4	1	1
Littorina keenae	Pacific Grove, California, USA	4	1	
	Big Sur, California, USA			1
Littorina scutulata	Pacific Grove, California, USA	3	1	–
Littorina plena	Candlestick Park, San Francisco, USA	3	1	1
Littorina squalida	Abashiri, Hokkaido, Japan	1	1	1
Littorina littorea	St Lawrence, Isle of Wight, England	6	1	1
	West Angle Bay, near Pembroke, Wales	3	1	1
	The Lizard, Cornwall, England			
Littorina kasatka	Akkeshi, Hokkaido, Japan	1	1	1
Littorina brevicula	Nagasaki Prefecture, Kyushu, Japan	1	1	1
Littorina mandshurica	Abashiri, Hokkaido, Japan	–	1	1
Littorina sitkana	Akkeshi, Hokkaido, Japan	1	1	1
Littorina horikawai	Tsutsu, Tsushima I., Japan	1	1	1
Littorina subrotundata	Charleston, Oregon, USA	3	1	1
Littorina aleutica	Provideniya, Russia	1	1	1
Littorina natica	Cape Kamchatskiy, Russia	–	1	–
Littorina obtusata	Pembroke Dock, Wales	3	1	1
Littorina fabalis	West Angle Bay, near Pembroke, Wales	3	1	1
Littorina compressa	West Angle Bay, near Pembroke, Wales	3	1	1
Littorina arcana	St Govan's Head, near Pembroke, Wales	3	1	
	The Lizard, Cornwall, England			
Littorina saxatilis	St Govan's Head, near Pembroke, Wales	2		
	St Lawrence, Isle of Wight, England	2		
	The Lizard, Cornwall, England		1	1
	Alberoni, Venice, Italy	2		
	Langebaan Lagoon, South Africa	4		
Mainwaringia rhizophila Reid, 1986	Sarimbun, Singapore	–	2	–
Nodilittorina angustior (Mørch, 1876)	Antigua	–	1	–
Nodilittorina dilatata (d'Orbigny, 1842)	Antigua	–	1	–
Nodilittorina punctata (Gmelin, 1791)	Tarhzoute, north of Agadir, Morocco	–	1	–
Nodilittorina radiata (Eydoux & Souleyet, 1852)	Cape d'Aguilar, Hong Kong	2	1	–
Nodilittorina trochoides (Gray, 1839)	Cape d'Aguilar, Hong Kong	2	1	–
Nodilittorina ziczac (Gmelin, 1791)	Antigua	–	1	–
Littoraria articulata (Philippi, 1846)	Sarimbun, Singapore	–	1	–
Littoraria intermedia (Philippi, 1846)	Lizard Island, Queensland, Australia	–	1	–
Littoraria pintado (Wood, 1828)	Oahu, Hawaii	–	1	–

synonym of *L. subrotundata* (Carpenter, 1864) (Reid & Golikov 1991). There is some controversy about the status of *L. neglecta* Bean, 1844; here it is regarded only as an ecotypic form of *L. saxatilis* (Olivi, 1792) (K. & B. Johannesson 1990; B. & K. Johannesson 1990; Reid 1993; Crossland *et al.* 1995) and has therefore been excluded. However, at least in some parts of its range it may be morphologically and physiologically distinct, so that some authors consider it a separate species (Grahame *et al.* 1995); whatever the outcome, the two are clearly very closely related. Three species must be added to the earlier list; two are newly described (*L. kasatka* Reid, Zaslavskaya & Sergievsky, 1991; *L. naticoides* Reid & Golikov, 1991, now renamed *L. natica* Reid, 1996), whereas a third (*L. horikawai* Matsubayashi & Habe, in Habe, 1979) has only recently been confirmed as a member of the genus (Reid 1996). Two purely nomenclatural changes must be mentioned, *L. nigrolineata* of authors, not Gray, 1839, should now be known as *L. compressa* Jeffreys, 1865, and *L. mariae* Sacchi & Rastelli, 1966, as *L. fabalis* (Turton, 1825). A recent monographic account

of the systematics and taxonomy of *Littorina* explains all these changes in detail (Reid 1996).

The 19 living species of *Littorina* currently recognized are listed in table 4, with authorities. Geographical distributions are also given (updating Reid 1990*b*; see Reid 1996, for detailed distribution records), providing necessary background information for the discussion of historical biogeography. The fossil record is summarized from Reid (1996).

(*b*) *Morphological phylogenetic analysis*

The starting point for the morphological analysis was that of Reid (1990*a*), but with the insertion, deletion and renaming of taxa as described above. During the preparation of detailed anatomical descriptions of all species (Reid 1996), the morphological characters utilized in the earlier study have been reevaluated and some additional characters included, giving a substantially different dataset (tables 2 and 3). Brief justifications for the changes are given in the Results section, but full descriptions appear in Reid (1996). Voucher material of all species is deposited in the Natural History Museum, London. As before, three species of the sister-genus (Reid 1989) *Nodilittorina* were used as the outgroup (*N. meleagris* (Potiez & Michaud, 1838), *N. dilatata* (d'Orbigny, 1842), *N. pyramidalis* (Quoy & Gaimard, 1833)). A Wagner parsimony analysis was performed using PAUP, version 3.1.1 (Swofford 1993). All characters were specified as unordered, and were unweighted. The HEURISTIC search option was employed, with ten repeats of a random addition sequence of taxa, and with the MULPARS option in effect. ACCTRAN optimization and OUTGROUP rooting were used. A strict consensus tree of all minimum-length trees was computed.

(*c*) *Molecular phylogenetic analysis*

(*i*) *Sample collection and DNA extraction*

The taxa sampled and their associated collection information are listed in table 1. Where possible, living material was used, and was anaesthetized and dissected as described by Rumbak *et al.* (1994). Samples preserved in pure ethanol were also satisfactory, and such material was repeatedly rinsed in sterile distilled water before processing. All DNA samples were extracted from single individuals. Tissue samples were disrupted by beating with glass beads, and total DNA was then extracted using the CTAB protocol of Doyle (1991), as modified by Winnepenninckx *et al.* (1993).

(*ii*) *Polymerase chain reaction and DNA sequencing*

A fragment of the mitochondrial small-subunit ribosomal RNA gene (12S rRNA) was amplified using the 'universal' primer pair 12Sa and 12Sb (Kocher *et al.* 1989), and a fragment of the large-subunit ribosomal RNA gene (16S rRNA) with the pair 16Sar and 16Sbr (Simon *et al.* 1991). Primers for the mitochondrial cytochrome-*b* gene were developed by alignment of the complete sequences of this gene in *Littorina littorea* (E. Rumbak, unpublished data), the pulmonate gastropod *Cepaea nemoralis* (Terrett *et al.* 1996), the insect *Drosophila yakuba* (Clary & Wolstenholme 1985), the echinoderm *Paracentrotus lividus* (Cantatore *et al.* 1989) and the nematode *Ascaris suum* (Okimoto *et al.* 1992). Two sets of primer pairs with a gastropod base composition bias were chosen from regions of high sequence conservation. No single pair of primers was able to amplify the cytochrome-*b* gene segment in all species examined, so that combinations of primers were used and the overlapping regions of amplified DNA compared. The primer sequences were as follows: primer 1: 5′-CCTTCCCGCACCTTCAAATC-3′ (14825); primer 2: 5′-TTGCAATACACTACACAG-3′ (14915); primer 3: 5′-GCAAATAAAAAGTATCACTCTGG-3′ (15554); primer 4: 5′-ATGAGAAATTTTCAG-GGTC-3′ (15515) (numbers in parentheses indicate the 3′ positions in the corresponding human mitochondrial sequence; Anderson *et al.* 1981). Amplifications were performed in a 100 µl volume containing 67 mM Tris-HCl (pH 8.8), 0.05% Tween-20, 100 µg ml^{-1} bovine serum albumen, 40 pmols of each primer, 100 µM of each dNTP, 2.5 units of *Taq* polymerase (Perkin-Elmer/Cetus), 10–100 ng of template DNA and concentrations of $MgCl_2$ between 0.5 and 2.0 mM. The $MgCl_2$ concentration was titrated for each set of primers to optimize the PCR yield. The cycling parameters for amplification were an initial denaturation for 5 min at 94 °C, and then 30 cycles of the following: denaturation for 0.75 min at 94 °C, 1.5 min at the annealing temperature (48–55 °C, depending on primer pairs) and 2 min extension time at 72 °C. Amplified products were extracted in chloroform, concentrated in a Centricon 30 columns (Amicon), and then directly sequenced using the thermocycling method described in the Taquence cycle-sequencing kit (United States Biochemical), at annealing temperatures dependent on the primers being used. The DNA sequence was determined for both strands, using the PCR primers and internal sequencing primers designed during the study. The sequences of the 12S gene for 11 *Littorina* species and for *Nodilittorina radiata* and *N. trochoides* have already been published (Rumbak *et al.* 1994) and have GenBank accession numbers U05862–U05874; the additional sequences indicated in table 1 have GenBank accession numbers U46784–U46835. Aligned sequence data are available from the authors.

(*iii*) *Sequence analysis and phylogeny reconstruction*

Sequences were aligned with the aid of the program CLUSTAL V (Higgins *et al.* 1992), with minor adjustments made by inspection. Base composition statistics and counts of transitions and transversions were done using a program by one of the authors (RHT). Stationarity of base composition was tested with programs by Rzhetsky & Nei (1995). Maximum likelihood tests of relative rates were conducted with the program 'codrates' (Muse & Weir 1992). Analyses using maximum-parsimony criteria were carried out with PAUP, version 3.1.1 (Swofford 1993). Bootstrapped distance-based analyses were done with

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHYLIP, version 3.52c (Felsenstein 1993) using Kimura's (1980) two-parameter distances and the neighbour-joining algorithm of Saitou & Nei (1987) for the construction of trees. Maximum-likelihood analyses (Felsenstein 1981) were done with PHYLIP, version 3.52c (Felsenstein 1993). Further details of methodology are given in the Results section.

(*iv*) *Evolutionary rates*

To calculate rates of molecular evolution from the DNA sequences, it is necessary to have estimates of the age of branching points on the phylogeny. Some of these were obtained from the fossil record, and others from geological and climatic events that are believed to have resulted in vicariance. In one case an estimate of age from a published allozyme study was used. Rates were calculated separately for both transitions and transversions throughout the sequences of the 12S and 16S genes, and in third positions only for the sequence of cytochrome-*b*. For each datable node, percentage pairwise differences for all taxa for which that node represented the last common ancestor were averaged, and the means regressed against the mid-points of the estimates of their ages. A linear regression model was used, with lines forced through the origin. The resulting regression equations, if significant, were used to estimate the ages of all clades. When estimating a molecular age in this way for a clade that had been used in the calculation of the regression, this point was excluded and the regression recalculated, to avoid circularity.

3. RESULTS

(*a*) *Morphological phylogeny*

The morphological characters are listed in table 2, and the complete character-state matrix for the cladistic analysis is given in table 3. The following changes have been made to the dataset of Reid (1990*a*). As noted above, three taxa have been removed and three added. The several autapomorphic characters of *Mainwaringia* have been removed. The shell shape and sculpture characters have been combined to give a single character. Four broad groups are here recognized, based on distinctive combinations of shell shape, spiral sculpture and colour pattern; further division cannot usefully be made because of the high degree of intraspecific variation in shell features. The colour pattern of the head has been removed as it is insufficiently consistent within species. It is now clear that the glandular projection of the penis seen in *L. scutulata* and *L. plena* is not homologous with the mamilliform penial glands of other species, giving a new character. The numbers of mamilliform glands have been recoded to take account of intraspecific variation. The position of the copulatory bursa is difficult to classify when the jelly gland is small, so this character has been omitted. To take account of intraspecific variation in the spiral arrangement of the egg groove of the pallial oviduct of *L. subrotundata*, the value for this species has been changed to state 4. A new character has been discovered, the additional first loop of the albumen gland. New data on the egg capsules of the subgenus *Neritrema* have become available, showing that an ovoid capsule is present in all but *L. saxatilis*. The number of eggs per capsule in the planktotrophic species is too variable to be used as a character. The type of development and ovoviviparity have been combined into a single character. New data from scanning electron microscopy of radulae have led to the reappraisal of the radular characters.

The analysis produced 21 equally parsimonious trees, each of 30 steps (consistency index = 0.933; retention index = 0.973; rescaled consistency index = 0.908). The strict consensus tree is shown in figure 1, with the unambiguous character state changes indicated. All characters were specified as unorded in this analysis. Ordering of characters 3, 6, 8 and 10 (for which there was some justification for recognizing transformation sequences) had only a small effect on the consensus tree, increasing the number of steps to 33, removing *L. kasatka* from the central polychotomy as the first branch, making *L. squalida* and *L. littorea* sister-species, and reducing all the *Littorina* (*Neritrema*) species to a polychotomy, within which *L. aleutica* and *L. natica* were sister-species, and the Atlantic species (*L. obtusata*, *L. fabalis*, *L. compressa*, *L. arcana*, *L. saxatilis*) formed a clade.

(*b*) *Fossil record*

The fossil record of living and extinct *Littorina* species is summarized in table 4; only eight are known from the Pliocene or earlier, and of these four are extinct. The extinct species were not included in the morphological phylogenetic analysis, as only two of the characters analysed relate to the shell. In no cases are clades or species defined by unambiguously synapomorphic shell characters, so that fossil shells can only be identified, and their relationships assessed, by subjective evaluation of overall resemblance to living species. The first appearance of a species is taken as a minimum age for the origin of the clade concerned (which involves the assumption that features used for recognition are apomorphic).

The earliest possible member of the genus is an unnamed species from the Palaeocene of Baja California, figured by Woods & Saul (1986). The generic assigment is uncertain, because the definition of the genus depends upon anatomical characters, and the shells of its basal members are not clearly separable from those of the sister-genus *Nodilittorina*. It is therefore unlikely that the age of the genus *Littorina* will ever be known from the fossil record. The earliest probable fossils of *Nodilittorina* are from the Middle Miocene (Reid 1989). The oldest certain member of *Littorina* is the fossil *L. sookensis* from the Upper Oligocene (23.5–30 Ma BP); this resembles only the living *L. keenae*, and therefore provides an estimate of the age of separation of the lineage leading to that species. The affinities of *L. remondii*, from the Upper Miocene, are less certain; it has been compared with *L. scutulata* and *L. plena* (Reid 1996), but does not provide useful phylogenetic information. Two Recent species first

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 2. *Characters used in the morphological phylogenetic analysis of* Littorina *species*

character no.	character	character state	description of character state
1	shell shape and sculpture	0	*Nodilittorina*-like
		1	*L. squalida*-like
		2	*Neritrema*-like
		3	*L. obtusata*-like
2	shell mineralogy	0	aragonite only
		1	outer calcite, inner aragonite layer
3	mamilliform penial glands	0	absent
		1	0 or 1 gland in same species
		2	2 glands
		3	many (3 or more) glands
4	glandular projection of penial filament	0	absent
		1	present (e.g. *L. plena*)
5	paraspermatozoa	0	rod-pieces present
		1	rod-pieces absent
6	egg groove of pallial oviduct	0	*Nodilittorina*-like
		1	*L. striata*-like
		2	*L. keenae*-like
		3	*L. littorea*-like
		4	*L. sitkana*-like
		5	*L. obtusata*-like
		6	*L. saxatilis*-like
7	albumen gland of pallial oviduct	0	single loop
		1	additional first loop (e.g. *L. aleutica*)
8	jelly gland of pallial oviduct	0	small (e.g. *L. littorea*)
		1	enlarged; thick glandular septa (e.g. *L. obtusata*)
		2	modified as brood pouch
9	egg capsules	0	pelagic, sculptured cupola (e.g. *Nodilittorina*)
		1	pelagic, biconvex
		2	pelagic, two rims (e.g. *L. scutulata*)
		3	within benthic gelatinous mass, ovoid
		4	retained in brood pouch, thin egg covering only
10	development	0	planktotrophic
		1	nonplanktotrophic
		2	ovoviviparous
11	outer marginal radular teeth	0	outer basal projection, long cusps (e.g. *L. keenae*)
		1	projection reduced, cusps shorter (e.g. *L. littorea*)
12	cusps of five central radular teeth	0	long, rectangular (e.g. *L. keenae*)
		1	short, pointed (e.g. *L. littorea*)
		2	short, blunt (e.g. *L. obtusata*)
13	salivary glands	0	small (e.g. *Nodilittorina*)
		1	enlarged (e.g. *L. littorea*)

appear in the Middle Miocene, *L. squalida* and *L. brevicula*; the former is slightly older and its age of 12–15 Ma gives a minimum estimate of the time of their separation. The Pliocene *L. petricola* is believed to be an extinct derivative of *L. squalida*, providing no information on the relationships of Recent species. The monophyly of the five Atlantic species of the subgenus *Neritrema* (*L. obtusata*, *L. fabalis*, *L. compressa*, *L. arcana* and *L. saxatilis*) is not unequivocally supported by the morphological analysis (although it is strongly supported by the molecular evidence, described below); a possible ancestor of the latter three (*L. islandica*) is known from 2 Ma BP, as is *L. fabalis*, estimating the minimum age of divergence among these five species.

(*c*) *Gene phylogeny*

Owing to methodological difficulties, it was not possible to obtain complete data for all three genes for all taxa in this study. Only for a region of the mitochondrial 16S rRNA gene are data available for all species. The alignment of these data contains 444 sites, of which 195 are variable and 115 are parsimony informative. Alignment of this region was for the most part unproblematic. An exception was a 50 b.p. region where insertion/deletion events in the *Mainwaringia* sequence relative to the rest gives rise to a run of alignment-ambiguous sites when that taxon is included. A tree resulting from a distance-based analysis is presented in figure 2; very similar topologies were recovered by other methods also (table 5). Visual inspection of the aligned sequence data and the branch lengths of the resulting distance tree clearly indicate that *Mainwaringia* is quite distantly related to the genus *Littorina*, and it will therefore be considered an outgroup in subsequent analyses. The most striking result is the apparently anomalous placement of the genus *Littoraria* as a sister-group to *Littorina striata*. The same placement is also significantly supported in both maximum-likelihood and maximum-parsimony analy-

Table 3. *Character state matrix for the morphological phylogenetic analysis of* Littorina *species*

(For description of characters and character states see table 2. The outgroup comprises three species of *Nodilittorina*. Unknown character states are indicated by a query.)

species	character number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
N. meleagris	0	0	0	0	0	0	0	0	0	0	0	0	0
N. dilatata	0	0	1	0	0	0	0	0	0	0	0	0	0
N. pyramidalis	0	0	1	0	0	0	0	0	0	0	0	0	0
L. striata	0	0	0	0	1	1	0	0	0	0	0	0	0
L. keenae	0	0	2	0	1	2	0	1	1	0	0	0	0
L. scutulata	0	1	0	1	1	3	0	0	2	0	0	0	1
L. plena	0	1	0	1	1	3	0	0	2	0	0	0	1
L. squalida	1	1	3	0	1	3	0	0	1	0	1	1	1
L. littorea	1	1	3	0	1	3	0	0	1	0	1	1	1
L. kasatka	2	1	1	0	1	3	0	0	?	0	1	1	1
L. brevicula	2	1	3	0	1	3	0	0	1	0	1	1	1
L. mandshurica	2	1	3	0	1	3	0	0	1	0	1	1	1
L. sitkana	2	1	3	0	1	4	0	1	3	1	1	1	1
L. horikawai	2	1	3	0	1	4	0	1	?	1	1	1	1
L. subrotundata	2	1	3	0	1	4	0	1	3	1	1	1	1
L. aleutica	2	1	3	0	1	3	1	1	3	1	1	2	1
L. natica	2	1	3	0	1	3	1	1	3	1	1	2	1
L. obtusata	3	1	3	0	1	5	0	1	3	1	1	2	1
L. fabalis	3	1	3	0	1	5	0	1	3	1	1	2	1
L. compressa	2	1	3	0	1	5	0	1	3	1	1	2	1
L. arcana	2	1	3	0	1	5	0	1	3	1	1	2	1
L. saxatilis	2	1	3	0	1	6	0	2	4	2	1	2	1

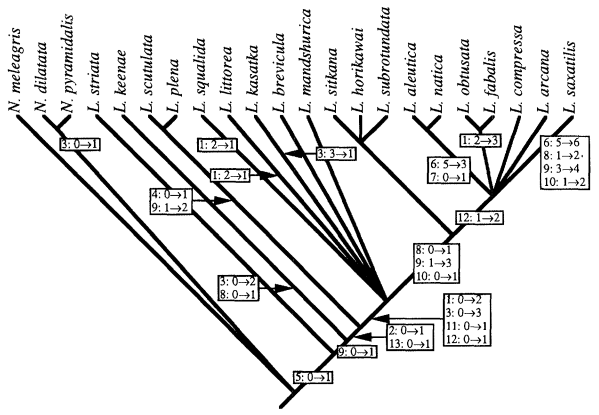

Figure 1. Morphological cladogram for the 19 living species of *Littorina*. This is the strict consensus tree of 21 equally parsimonious trees derived from characters and character states given in tables 2 and 3. Boxes indicate only unambiguous character state changes (in the format 'character number: state change').

ses (results not shown). Several lines of evidence support the conclusion that this placement of *Littoraria* is an artefact arising from compositional biases in the data. Inspection of base-composition and skewness measures (Perna & Kocher 1995) (figure 3) shows that *Littoraria* and *Littorina* are very similar, in contrast to *Mainwaringia* and *Nodilittorina*, indicating a shared tendency to have excess G's and T's on the same strand (relative to equal occurrence on both strands). Stationarity of base composition is assumed by the available analytical methods. Tests of this assumption by the method of Rzhetsky & Nei (1995) show no significant heterogeneity of base composition within any of these genera. *Littorina* and *Littoraria* species considered together also show no significant heterogeneity, but highly significant heterogeneity is seen in the combined *Nodilittorina* and *Littoraria* species. Thus there is a systematic bias towards *Littorina* and *Littoraria* grouping together. Inspection of the branch lengths for *Littoraria* in figure 2 suggests marked rate heterogeneity if this topology is accepted. A maximum-likelihood relative-rate method (Muse & Weir 1992) was used to test for heterogeneity of evolutionary rates over the topology shown in figure 2 and over that expected on morphological grounds, in which *Littoraria* is an outgroup to the sister-genera *Littorina* and *Nodilittorina* (Reid 1989). Taking *Nodilittorina* as an outgroup, very little or no significant rate heterogeneity is observed within either *Littorina* or *Littoraria*, whereas comparisons involving species from both ingroup genera virtually always show significant rate heterogeneity. With *Littoraria* taken as the outgroup, very little or no significant rate heterogeneity is observed within either *Littorina* or *Nodilittorina*. Comparisons involving species from both these ingroup genera show significant rate heterogeneity in only about a quarter of comparisons. Thus acceptance of the morphological topology requires little rate heterogeneity in the 16S data when compared to the tree recovered by the usual methods of sequence analysis. Given the support from morphological data for the sister-group relationship of *Littorina* and *Nodilittorina*, we prefer to regard the placement of *Littoraria* as a sister-group to *L. striata* by analyses of the 16S data as an artefact. The 16S data do provide strong support for the monophyly of the genus *Littoraria* and for that of *Nodilittorina*. If *Littoraria* is excluded, the monophyly of *Littorina* is also supported.

Preliminary results from a fragment of the mitochondrial small subunit (12S) ribosomal RNA gene have been reported previously (Rumbak *et al.* 1994). Here, additional taxa are added to the analysis (table 1). The alignment consists of 374 sites, of which 136 are variable and 87 are parsimony informative. Secondary structure predictions for this domain 3 region were used to guide the alignment and few alignment-ambiguous sites remain. These data show no significant departure from stationarity of base composition according to the test of Rzhetsky & Nei (1995). Phylogenetic resolution, as measured by bootstrap support (table 5), is strongest for the most basal taxa in *Littorina*, *L. striata* and then *L. keenae*. Some pairs of terminal sister-species also receive support.

The 651 b.p. fragment of the mitochondrial cytochrome-*b* gene contains 318 variable sites and 193 parsimony-informative sites. No difficulties were encountered in the alignment of these data, because no insertion/deletion events were required. First and second codon-position sites, corresponding roughly to nonsynonymous sites, show no significant heterogeneity of base composition (Rzhetsky & Nei 1995), whereas third position sites, which are almost all synonymous, do show significant heterogeneity. Compared with the

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Table 4. *Summary of geographical distribution and fossil record of* Littorina *species*

(For Recent species, geographical distribution is the modern range, for extinct species it is the entire geographical range of fossil records. Data from Reid 1996.)

species	geographical distribution	fossil record	references to earliest fossil occurrence
Littorina sp., undescribed	Baja California	Palaeocene	Woods & Saul 1986
Littorina (*Liralittorina*) *striata* King & Broderip, 1832	Azores, Madeira, Canary Is, Cape Verde Is	Pleistocene to Recent	Reid 1996
[a]*Littorina* (*Planilittorina*) *sookensis* Clark & Arnold, 1923	Vancouver I.	Upper Oligocene	Clark & Arnold 1923
Littorina (*Planilittorina*) *keenae* Rosewater, 1978	Oregon to Baja California	Pleistocene to Recent	Grant & Gale 1931
[a]*Littorina* (*Littorina*) *remondii* Gabb, 1866	Central California	Upper Miocene	Clark 1915
Littorina (*Littorina*) *scutulata* Gould, 1849	SE Alaska to Baja California	Pliocene to Recent	Reid 1996
Littorina (*Littorina*) *plena* Gould, 1849	Gulf of Alaska to California	not known	
Littorina (*Littorina*) *squalida* Broderip & Sowerby, 1829	N Sea of Japan to Bering Strait	Middle Miocene to Recent	Gladenkov & Sinelnikova 1990 (as *L. praesqualida*)
[a]*Littorina* (*Littorina*) *petricola* Arnold, 1908	California and Oregon	Pliocene	Woodring et al. 1940 (as *L. mariana*)
Littorina (*Littorina*) *littorea* (Linnaeus, 1758)	White Sea to Portugal, Newfoundland to Virginia	Upper Pliocene to Recent	Harmer 1921
Littorina (*Littorina*) *kasatka* Reid, Zaslavskaya & Sergievsky, 1991	Kamchatka to Hokkaido	not known	
Littorina (*Littorina*) *brevicula* (Philippi, 1844)	Japan, Korea, China	Middle Miocene to Recent	Gladenkov & Sinelnikova 1990 (as *L. itelmenica*)
Littorina (*Littorina*) *mandshurica* (Schrenck, 1861)	N Sea of Japan	Pleistocene to Recent	Reid 1996
Littorina (*Neritrema*) *sitkana* Philippi, 1846	N Pacific, from Oregon to Bering Sea to N Sea of Japan	not known	
Littorina (*Neritrema*) *horikawai* Matsubayashi & Habe, in Habe, 1979	Islands off Kyushu	not known	
Littorina (*Neritrema*) *subrotundata* (Carpenter, 1864)	N Pacific from N California to Aleutian Is to Kurile Is	not known	
Littorina (*Neritrema*) *aleutica Dall*, 1872	Aleutian Is, Bering Sea	not known	
Littorina (*Neritrema*) *natica* Reid, 1996	Kurile Is, Kamchatka, NW Bering Sea	not known	
Littorina (*Neritrema*) *obtusata* (Linnaeus, 1758)	White Sea to Portugal, Iceland, Greenland, Labrador to Long I.	Pleistocene to Recent	Reid 1996
Littorina (*Neritrema*) *fabalis* (Turton, 1825)	White Sea to Portugal, Iceland, SW Greenland	Upper Pliocene to Recent	Neuville & Ruhlmann 1941
[a]*Littorina* (*Neritrema*) *islandica* Reid, 1996	Iceland	Upper Pliocene	Reid 1996
Littorina (*Neritrema*) *compressa* Jeffreys, 1865	Barents Sea, British Isles, N France	not known	
Littorina (*Neritrema*) *arcana* Hannaford Ellis, 1978	N Norway, British Isles N France	not known	
Littorina (*Neritrema*) *saxatilis* (Olivi, 1792)	Novaya Zemlya to Morocco, Venice, Tunisia, Canary Is, Azores, S Africa, Iceland, Greenland, Baffin I. to Hudson Bay and Chesapeake Bay	Pleistocene to Recent	Lecointre 1952

[a] Extinct species.

two ribosomal RNA genes, cytochrome-*b* provides enhanced resolution within the genus *Littorina* (table 5), although once again only the more basal and the more terminal branches receive strong support.

Sixteen *Littorina* species have been sequenced for all three gene fragments (table 1) and a maximum-parsimony tree obtained from the combined dataset is given in figure 4. The tree is shown rooted with *L.*

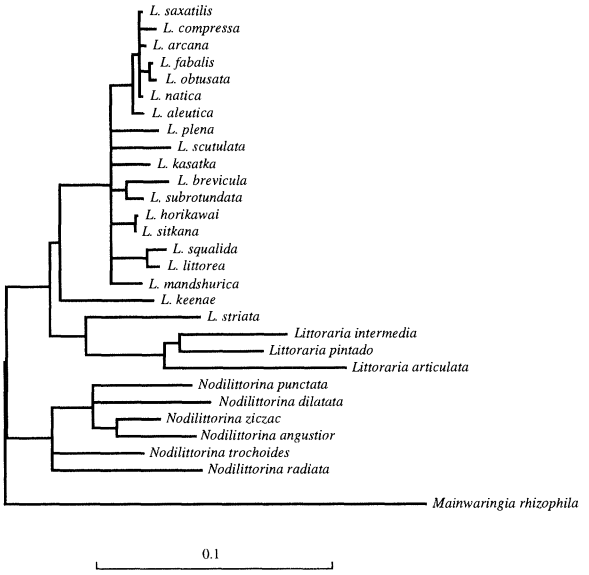

Figure 2. Neighbour-joining tree based on DNA base sequence of the mitochondrial 16S ribosomal RNA gene, for *Mainwaringia rhizophila*, 6 *Nodilittorina* species, 3 *Littoraria* species and all species of *Littorina*. Tree has been constructed from pairwise distances calculated using Kimura's (1980) two-parameter correction for multiple substitutions. Branch lengths are scaled in terms of the expected numbers of substitutions. This is a bootstrap 50% majority-rule consensus tree based on 100 replicates; groups with less than 50% support have been collapsed to polychotomies (see table 5 for bootstrap values). *Mainwaringia rhizophila* has been used as an outgroup to root the tree. Note the anomalous position of the genus *Littoraria*.

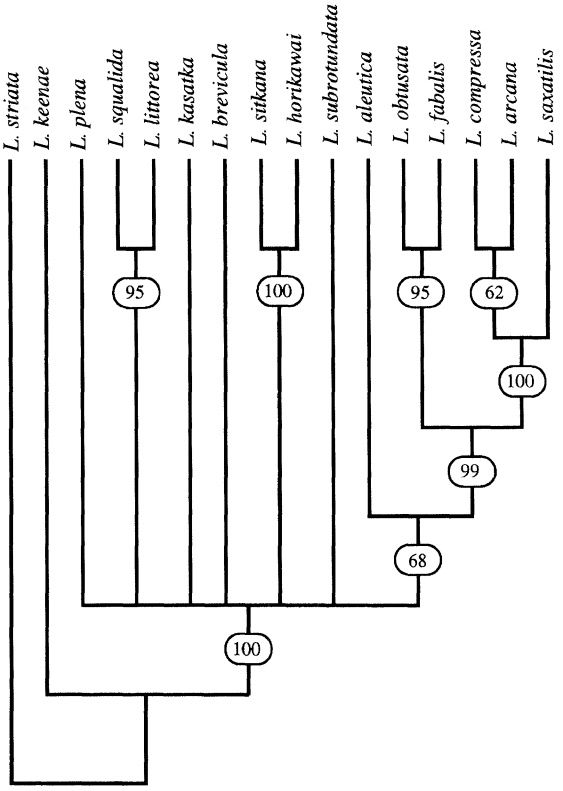

Figure 4. Maximum-parsimony tree based on DNA sequences of the 12S, 16S and cytochrome-*b* mitochondrial genes (totalling 1469 bp) for 16 *Littorina* species. Tree produced with PAUP (Swofford 1993) using simple addition sequence and TBR branch swapping; all minimal-length trees were saved (MULPARS option); zero-length branches were collapsed. There are 395 informative sites and 576 character states, producing a tree of 1026 steps. This is a bootstrap 50% majority-rule consensus tree based on 100 replicates; numbers in ellipses are percentages of replicates in which taxa occur together; groups with less than 50% support have been collapsed to polychotomies. *Littorina striata* has been used as an outgroup to root the tree.

striata, as supported by the 12S and 16S analyses. The tree topology is similar to that obtained from the cytochrome-*b* data, but with higher bootstrap support for some clades. As in each of the individual gene-fragment analyses, a large central polychotomy remains unresolved.

To summarize the results of the molecular phylogenetic analyses, the clades supported by bootstrap values greater than 50% in any of the analyses are listed in table 5. For comparison, the support for the same nodes in the morphological phylogeny is also given. In general, there were very few cases of contradictory topologies among the several analyses. To arrive at a consensus topology from the molecular analyses (the topology shown in figure 6), a tree based on the combined analysis of all three gene fragments (figure 4) has been taken as a starting point, and the

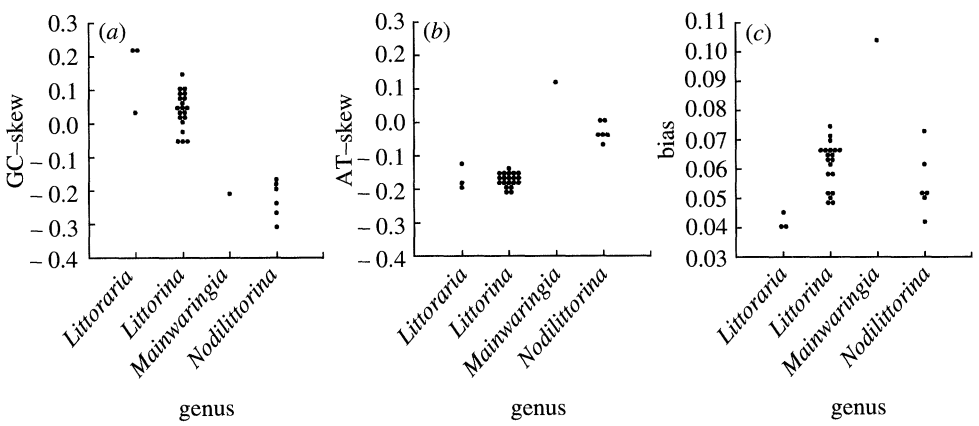

Figure 3. Base-composition, skewness and bias measures (Perna & Kocher 1995) for variable sites in the 16S data grouped by genus. (*a*) GC-skew. (*b*) AT-skew. (*c*) Bias.

Table 5. *Summary of support for clades of* Littorina *species from molecular phylogenetic analyses*

(All clades which in any of the molecular analyses had bootstrap values exceeding 50% are listed. For comparison, the results of the morphological analysis are included. Figures tabulated are bootstrap values for maximum-parsimony analysis and (following slash) for distance analysis. Key: eq, equivocal support in morphological analysis (supported only by alternative optimizations of character state changes, or by analysis in which some characters were ordered); no, topology contradicts clade; un, clade unresolved; yes, unequivocal support for clade in morphological analysis; –, not applicable. Molecular data were missing for some species, see table 1.)

clade (arbitrary number: *Littorina* species included)	12S gene	16S gene	cyt-*b* gene	all genes combined	morphological analysis
1: *compressa* + *arcana*	un/un	un/un	69/85	62/90	un
2: 1 + *saxatilis*	74/84	un/un	99/100	100/100	un
3: *obtusata* + *fabalis*	60/84	65/75	74/98	95/100	yes
4: 2 + 3	un/un	un/un	90/99	99/100	eq
5: 2 + 3 + *natica*	–	78/65	–	–	no
6: *aleutica* + *natica*	–	no/no	–	–	yes
7: 4 + *aleutica* (+ *natica* for 16S and morphological analyses)	un/no	75/51	67/87	68/84	yes
8: *sitkana* + *horikawai*	88/80	78/87	100/100	100/100	un
9: 8 + *subrotundata*	no/no	un/no	51/un	un/un	eq
10: 7 + 9 (i.e. subgenus *Neritrema*)	un/no	un/no	un/54	un/un	yes
11: *brevicula* + *mandshurica*	–	un/no	99/100	–	un
12: *littorea* + *squalida*	90/92	95/99	un/59	95/99	eq
13: 10 + 11 + 12 + *kasatka* (*mandshurica* not available for 12S)	un/no	un/no	un/no	un/un	yes
14: *scutulata* + *plena*	un/no	70/un	–	–	yes
15: 13 + 14 (*scutulata* not available for cyt-*b*)	95/99	91/99	99/100	100/100	yes
16: *keenae* + 15	91/92	71/50	–	–	yes
17: *striata* + 16	100/100	no/no	–	–	yes
18: *aleutica* + *subrotundata*	76/91	no/un	no/no	no/no	no
19: *scutulata* + 8	un/51	no/un	–	–	no
20: *brevicula* + *subrotundata*	no/no	un/54	no/no	un/un	no

three missing species (for which not all sequences were available) have been added as follows. *Littorina scutulata* is accepted as the sister-species of *L. plena*, on the basis of a bootstrap value of 70% in the 16S parsimony analysis (contradiction of this placement by the topology of clade 19 was only weakly supported by a bootstrap value of 51% in the 12S distance analysis). *Littorina mandshurica* is the sister-species of *L. brevicula*, as strongly supported by the bootstrap values of 99% and 100% in the parsimony and distance analyses respectively of the cytochrome-*b* data (and only weakly contradicted by 54% support for clade 20 in the 16S distance analysis). The position of *L. natica* is more problematic. In the 16S analyses there is reasonable support (78% and 65% in parsimony and distance analyses respectively) for this species as the sister-taxon to the five Atlantic members of the subgenus *Neritrema* (clade 4). However, this is contradicted by the morphological analysis, which identifies two unequivocal synapomorphies (one of them, character 7, unique) of *L. natica* and *L. aleutica* (figure 1). This alternative topology increases the length of the 16S parsimony tree by only a single step, and we therefore prefer to show the sister-taxon relationship of *L. natica* as unresolved, pending additional molecular data. Similarly, little confidence can be placed in the resolution of the three species of the *L. saxatilis* group (*L. saxatilis*, *L. arcana*, *L. compressa*), for the two alternative topologies to the one shown in figure 4 are but one step longer; this clade is therefore shown as an unresolved trichotomy in the consensus of the molecular analyses (topology of figure 6). These three species are so closely related that intraspecific variation is likely to be at least as great as that observed among the individuals sequenced here.

(*d*) *Rates of molecular evolution*

Five calibration points were available for most of the regressions of sequence divergence with time. The three estimates derived from the fossil record (for clades 4, 15 and 16; see figure 7 and table 5 for numbering of clades) are mentioned above. The two further points are based on the assumption that the two cases of sister-taxa in the northern Pacific and northern Atlantic diverged following the same vicariant event. The maximum age of this event is the opening of the Bering Strait, estimated at 3.5–4 Ma BP (Hopkins 1967), and the minimum the effective closure of the trans-Arctic migration route by climatic cooling, estimated at 2.4 Ma BP (when widespread glaciation was initiated; Shackleton *et al.* 1984; Loubere 1988). One of the cases of Pacific-Atlantic sister-taxa is the pair *L. squalida* and *L. littorea* (clade 12). The other is *L. aleutica* and *L. natica* in the Pacific and the five Atlantic *Neritrema* species (*L. obtusata*, *L. fabalis*, *L. compressa*, *L. arcana*, *L. saxatilis*) (clade 7, with an unresolved basal trichotomy). Inspection of the points suggested that the sequences for 12S and 16S were not saturated with

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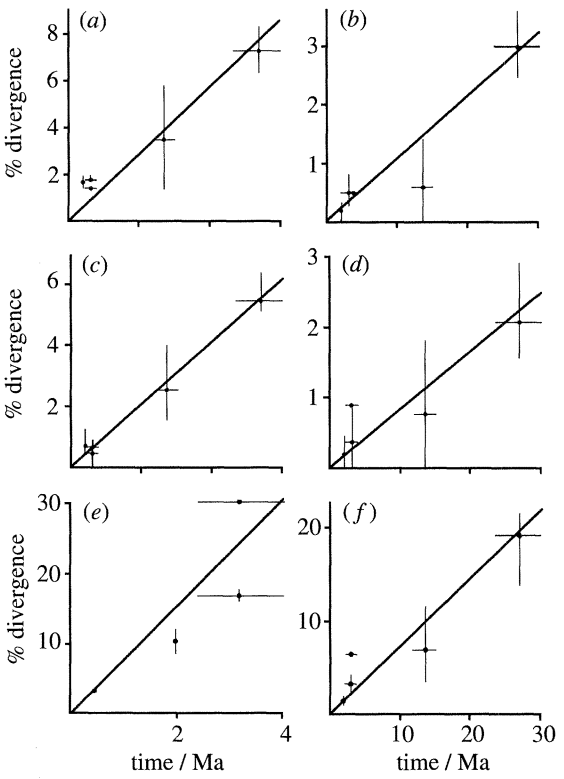

Figure 5. Rates of accumulation of transitions and transversions respectively in the 12S ((*a*) and (*b*)), 16S ((*c*) and (*d*)) and cytochrome-*b* ((*e*) and (*f*)) mitochondrial genes of *Littorina*. These rates have been used in the estimation of ages of clades (table 6, figure 6). Coefficients for the regressions of age on percentage divergence (for the estimation of unknown ages) are: 12S transitions = 3.495; 12S transversions = 9.195; 16S transitions = 4.895; 16S transversions = 11.941; cytochrome-*b* third position transitions = 0.131; cytochrome-*b* third position transversions = 1.358 ($P < 0.01$ for all regressions). Horizontal error bars show the range of estimates of the ages of calibration points (fossils and the Bering Strait); vertical error bars are the ranges of percentage difference in all possible pairwise comparisons of taxa of the same age.

respect to either transitions or transversions over the range of calibration, and this was also the case for third-position transversions in the cytochrome-*b* sequence. For third-position transitions in the cytochrome-*b* sequence, however, the two oldest points were within the range of saturation, and were therefore excluded from the regression calculation. In this case a fourth point was based on the 0.34–0.55 Ma BP age of separation between *L. arcana* and *L. saxatilis*, estimated from allozyme divergence by Knight *et al.* (1987); this was used as an age for the clade comprising these two species plus *L. compressa*.

The regressions of sequence divergence with time are plotted in figure 5, and the resulting molecular estimates of the ages of clades are summarized in table 6. These ages have been used in figure 6 to provide a timescale for the molecular phylogeny. The ranges of molecular estimates of the ages of clades are widest in the cases of sister-species pairs (clades 8, 11 and 14). This is to be expected as a sampling effect, because for sister-species pairs there is only a single value of transition or transversion difference from which to estimate age, whereas for polychotomies each age estimate is the result of averaged divergences among members of the clade. Although the final column in table 6 presents averages of the estimated ages for each clade, the individual estimates are not all equally reliable, owing to sampling effects where small numbers of substitutions are involved (where recent divergences are estimated from slow rates of substitution). Nevertheless, for the five points used for calibration of the rates of molecular divergence, the molecular estimates of age do overlap the independently derived estimates based on palaeontological and geological evidence.

4. DISCUSSION AND CONCLUSIONS

(*a*) *Towards a consensus phylogeny*

The morphological phylogeny presented here (figure 1) is in many ways similar to the earlier version of Reid (1990*a*), despite the addition and removal of taxa, and other modifications of the dataset described above. In each cladogram the first branch within the monophyletic clade *Littorina* is *L. striata*, followed by *L. keenae* and the sister-species pair *L. scutulata* and *L. plena*, and

Table 6. *Estimates of ages of clades in the phylogeny of* Littorina

(These ages (Ma) are derived from the fossil record, the geological age of the Bering Strait and from the regressions of sequence divergence with time (figure 5). Clades are numbered as in table 5; clades with limited support have been excluded (see text). ns = regression for the estimation of age of this clade not significant ($P < 0.05$), sat = saturated, ts = transitions, tv = transversions.)

clade	fossils or geology	12S, ts	12S, tv	16S, ts	16S, tv	cyt-*b*, 3rd ts	cyt-*b*, 3rd tv	mean of DNA ages
2	–	0	3.31	1.81	3.55	ns	0	1.73
3	–	3.49	0	1.08	2.63	0.72	0	1.32
4	2	5.99	1.65	3.70	2.27	1.31	2.08	2.83
7	2.4–4	6.36	5.11	2.30	4.57	1.95	4.43	4.12
8	–	3.85	12.69	3.28	0	0.97	3.12	3.99
11	–	–	–	15.62	2.63	1.99	3.12	5.84
12	2.4–4	4.94	5.11	3.28	10.07	5.72	9.38	6.42
14	–	18.17	2.48	11.94	18.63	–	–	12.81
15	12–15	11.94	5.74	12.39	8.72	sat	9.20	9.60
16	23.5–30	22.00	ns	28.11	ns	sat	23.70	25.92
17	–	39.90	40.60	32.47	43.93	sat	sat	39.23

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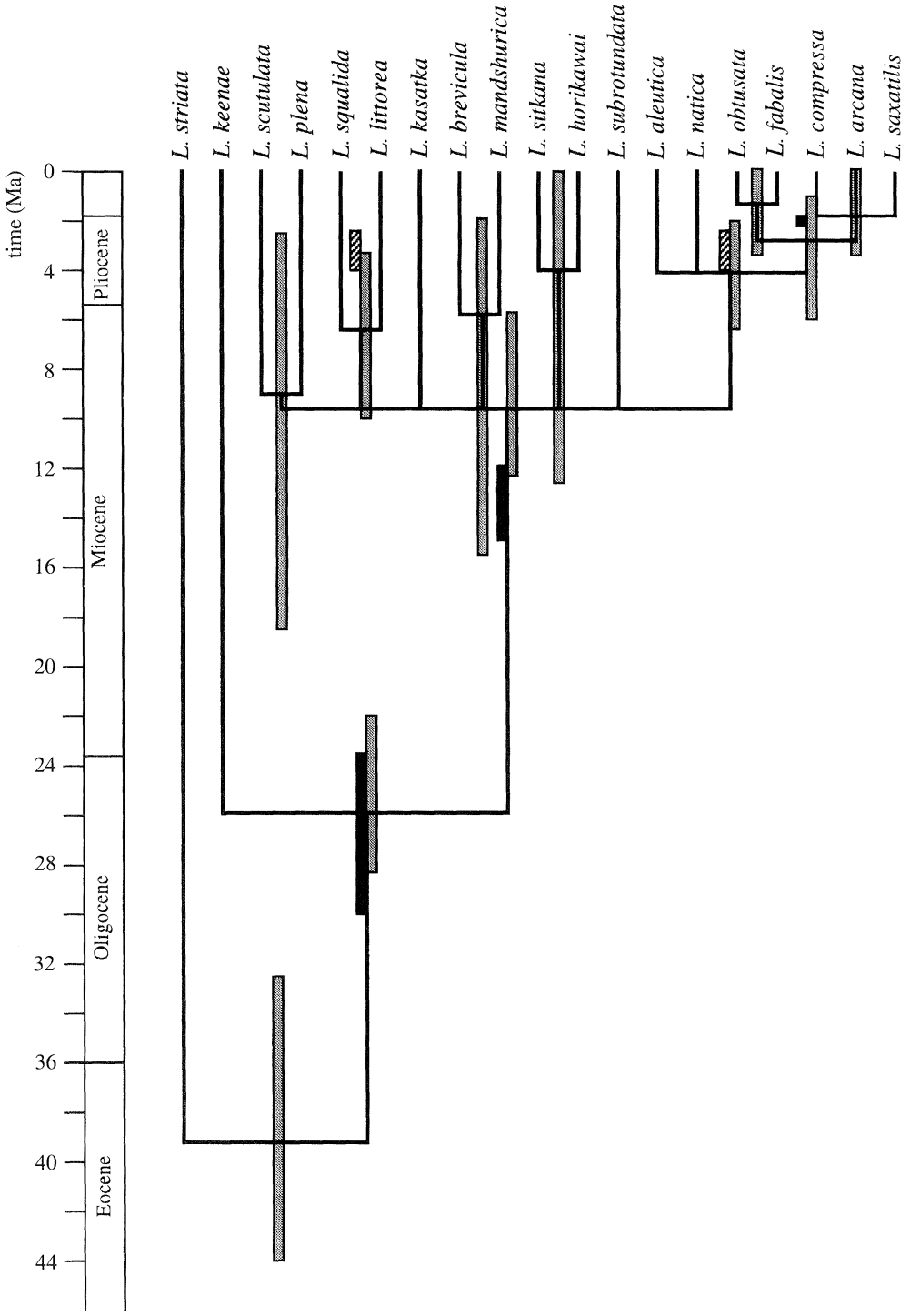

Figure 6. Phylogenetic tree of *Littorina* species, summarizing analyses of DNA base sequences of the 12S, 16S and cytochrome-*b* mitochondrial genes (topology derived from figure 4, with addition of three remaining taxa as described in text), with a timescale calibrated from fossil and geological evidence. Solid error bars indicate palaeontological estimates of age (the oldest fossil member of the clade). Hatched error bars indicate geological estimates of age (the estimated age of the opening of the Bering Strait, Hopkins 1967). These palaeontological and geological estimates of age have been used to calibrate the rates of molecular divergence (figure 5). Stippled error bars indicate the range of estimates of ages of each clade, based on rates of accumulation of transitions or transversions for each of the three genes (table 6). The origins of the clades are shown at the means of the molecular estimates of age (except for the pair *L. scutulata* and *L. plena*, where the mean age of their divergence was older than that of the polychotomy from which they arose).

in each the species of the nonplanktotrophic group (subgenus *Neritrema*) form a monophyletic clade. However, in the revised version, relationships among the remaining planktotrophic species (*L. squalida*, *L. littorea*, *L. kasatka*, *L. brevicula*, *L. mandshurica*) are not resolved, and these form a polychotomy with *Neritrema*. Another significant change is that, within the *Neritrema* clade, *L. aleutica* and *L. natica* form a monophyletic

group together with the five members of the subgenus from the Atlantic (*L. obtusata*, *L. fabalis*, *L. compressa*, *L. arcana*, *L. saxatilis*).

Turning to the molecular phylogenetic analyses, there is in general a high degree of agreement among the analyses of the DNA-sequence data from individual genes, irrespective of the gene and of the method of analysis. Although the degree of resolution varies, being greatest for the cytochrome-*b* gene, cases of contradiction are few (table 5). Unfortunately, because of methodological difficulties, it was not possible to obtain a complete dataset of all three gene sequences for all 19 *Littorina* species and appropriate outgroups. Accordingly, a two-step approach was used to summarize the molecular results. The first step was to analyse just those 16 *Littorina* species for which all three gene sequences were available. When rooted with *L. striata*, the resulting topology (figure 4) was entirely consistent with that from the morphological data. The topologies were identical in the basal position of *L. keenae*, and in the monophyly of *L. aleutica* plus the five Atlantic *Neritrema* species. In the molecular tree, phylogenetic resolution was enhanced in the terminal pairings of *L. squalida* with *L. littorea*, and of *L. sitkana* with *L. horikawai*, and also in the relationships among the Atlantic *Neritrema* species. However, resolution was poorer than in the morphological tree in the large central polychotomy, in which neither the basal position of *L. plena*, nor the monophyly of the subgenus *Neritrema*, were strongly supported. In the second step, the three species for which complete datasets were not available were added to the molecular tree, in positions supported by the results of the analyses of individual genes (figure 6). Thus the terminal pairings of *L. scutulata* with *L. plena*, and of *L. brevicula* with *L. mandshurica*, were established, again consistent with the morphological results and, in the case of the latter pairing, improving the resolution. Conflict occurred only in the position of *L. natica*, identified as the sister-taxon of the Atlantic *Neritrema* species by the 16S analysis, but as the sister-species of *L. aleutica* in the morphological analysis. In summary, therefore, the molecular analyses were largely consistent with the morphological one, showing increased resolution of recent divergences, and poorer resolution of a major polychotomy involving all species except *L. striata* and *L. keenae*. Because this polychotomy is a feature of both analyses, it may represent a real event of rapid cladogenesis in the evolution of the genus, and not simply a lack of phylogenetically useful characters in the morphological dataset.

Another approach to the reconstruction of phylogenetic relationships among *Littorina* species has been the analysis of gene frequencies, as estimated by allozyme electrophoresis. The use of different sets of loci and electrophoretic methods, and of alternative distance measures and clustering techniques, have sometimes produced divergent hypotheses of relationships (Sundberg *et al.* 1990; Backeljau & Warmoes 1992; Backeljau *et al.* 1994). However, at least among closely related species, for which this approach is most suitable, the results show some consistency and compatibility. So far, such studies have included only up to eight members of the genus. Ward (1990) analysed 16 to 25 loci of the six species in the northern Atlantic, showing *L. littorea* to be distantly related to the cluster of five *Neritrema* species, within which there were two clusters, the *L. obtusata* group (*L. obtusata* and *L. fabalis* [= *L. mariae*]) and the *L. saxatilis* group (*L. saxatilis* as the sister-species of *L. arcana*, and *L. compressa* [= *L. nigrolineata*]). This topology of the *L. saxatilis* group has also been recovered in a similar analysis of 22 loci of these three species alone (Knight & Ward 1991) and in a study including some Pacific species (Zaslavskaya *et al.* 1992); it is also the same as that derived from an analysis of the banding patters of random-amplified polymorphic DNAs (Crossland *et al.* 1995). Most of these allozyme studies have employed the method of starch-gel electrophoresis, and UPGMA clustering of Nei's (1972) genetic distances. More recently, Backeljau & Warmoes (1992) have used polyacrylamide-gel electrophoresis, which may detect more allelic variation, and have investigated the effects on tree topology of employing different measures of genetic distance and rate-independent clustering methods. Whereas UPGMA clustering of Nei's distances calculated from just nine loci recovered the standard topology of the *L. saxatilis* group, the authors advocated the use of the rate-independent distance-Wagner algorithm and Wright's Prevosti distances, from which *L. arcana* and *L. compressa* appeared as sister-species. This alternative topology is the same as that derived from the combined sequence data from the three mitochondrial genes presented here (figure 4). Most of the allozyme studies are in agreement that *L. obtusata* and *L. fabalis* are sister-species (Ward 1990; Backeljau & Warmoes 1992; although Zaslavskaya *et al.* 1992, linked *L. obtusata* more closely with *L. saxatilis*), as found by both morphological and DNA analyses in this study.

One of the few allozyme studies to have included examples of both Pacific and Atlantic *Littorina* species was that of Zaslavskaya *et al.* (1992). In two separate analyses using 13 and 17 loci, they showed that *L. sitkana* and three Atlantic *Neritrema* species formed a cluster, with the pair *L. brevicula* and *L. mandshurica* more distantly related, and the pair *L. squalida* and *L. littorea* appearing as an outgroup to the rest. This topology is consistent with those from the morphological and DNA analyses, but shows greater resolution, by linking the pair *L. mandshurica* and *L. brevicula* with the *Neritrema* cluster. The result is consistent with the monophyly of *Neritrema*, and of the Atlantic members of this subgenus, but provides a poor test of these relationships, because only four species of *Neritrema* were included. More recently, Zaslavskaya (1995) has analysed four Pacific species of *Littorina* and again showed the three members of *Neritrema* (*L. subrotundata*, *L. sitkana* and an unidentified third species) to be more closely related to each other than to the fourth species (*L. kasatka*). A different group of three species from each ocean was studied by Boulding *et al.* (1993). Only ten loci were assayed, of which six were invariant or showed low levels of variability. The resulting distance-Wagner tree showed a number of inconsistencies with the topologies described above,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

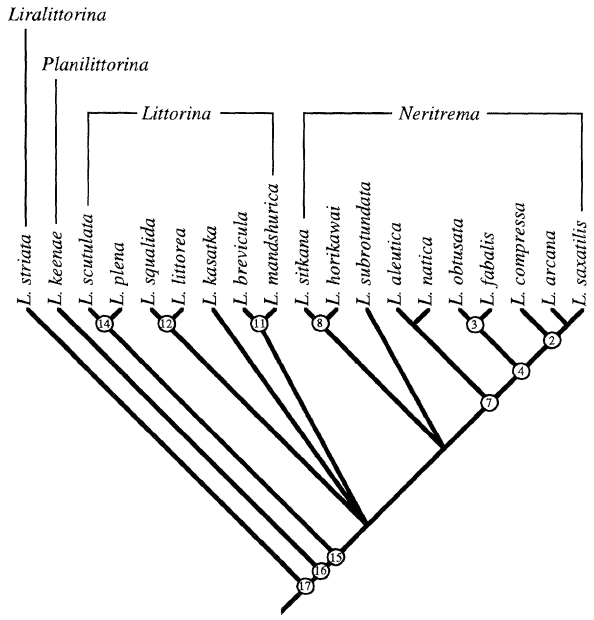

Figure 7. Consensus phylogeny of *Littorina* species, based on molecular and morphological analyses. Clades supported by the molecular analyses are numbered as in tables 5 and 6; the remaining clades are supported by morphological data or (in the case of *L. saxatilis* plus *L. arcana*) by allozyme data. The subgenera of *Littorina* (Reid 1990*a*, 1996) are superimposed.

but bootstrap values were generally low. In fact, if branches with bootstrap support below 50% were to be collapsed, and the tree re-rooted on *L. scutulata*, *L. littorea* would appear as the first branch, followed by *L. sitkana*, and, as a polychotomy, *L. subrotundata* (including *L.* sp. and *L.* '*kurila*' of these authors, see Reid 1996), *L. obtusata* and *L. saxatilis*. This is in close agreement with other topologies and does not contradict the monophyly of *Neritrema*.

Another potential source of evidence for phylogenetic relationships is the fossil record, although this is always open to the criticism of incompleteness (review by Smith & Littlewood 1994). It is unfortunate that in the case of *Littorina* the fossil record is poor, and furthermore that there are insufficient characters of the shell to permit inclusion of fossils in the same morphological analysis as the Recent species. Rigorous stratigraphic tests of phylogenetic hypotheses (e.g. Wagner 1995) are therefore not possible. A phylogenetic tree is an hypothesis of the chronological order of appearance of its clades. Assuming that the subjective evaluation of the relationships of the fossil examples are correct, it is notable that the order of stratigraphic appearance of the fossils *L. sookensis*, *L. squalida*, and the contemporaneous *L. islandica* and *L. fabalis*, is the same as the order of their presumed Recent representatives (*L. keenae*, *L. squalida* and the clade of Atlantic *Neritrema* species, repectively) in both morphological and molecular phylogenies.

It is therefore clear that there is a large measure of agreement between the different sources of phylogenetic information (morphology, mitochondrial gene sequences, allozyme frequencies and fossils) so that some kind of consensus can be attempted. There has been much recent debate about the appropriate phylogenetic treatment of different datasets, with some authors favouring their combination in a single dataset, to maximize descriptive and explanatory power (the 'character congruence' or 'total evidence' view, e.g. Eernisse & Kluge 1993). However, we adopt the alternative recommendation for separate analyses, because congruent topologies derived from independent datasets provide the best estimate of the true phylogeny ('taxonomic congruence', see review by Miyamoto & Fitch 1995). Various consensus procedures could be used to summarize the agreement among the datasets. Our preferred solution is shown in figure 7. This is essentially a combinable components consensus tree (which retains more information than a strict consensus tree) of the morphological (figure 1) and molecular (figure 6) trees, but with two small modifications. The monophyly of *L. sitkana*, *L. horikawai* and *L. subrotundata* is supported only by an ambiguous morphological synapomorphy, and has therefore not been recognized. The three members of the *L. saxatilis* group are clearly very closely related, and little confidence can be placed in the resolution derived from the molecular data, as discussed above; instead, the topology produced by the majority of the allozyme analyses is preferred (showing *L. saxatilis* and *L. arcana* as sister-species). It is notable that the additional resolution of the central polychotomy derived from the morphological tree is not contradicted by the molecular analysis. Specifically, the constraint of a monophyletic *Neritrema* and/or that of the monophyly of all *Littorina* species except *L. striata*, *L. keenae*, *L. scutulata* and *L. plena*, does not increase the length (940 steps) of the most parsimonious trees found in the parsimony analysis of the combined molecular dataset (i.e. of the 16 species for which all three gene sequences were available).

(*b*) *Systematic implications*

Despite advances in the knowledge of the comparative anatomy of littorinids, and the introduction of cladistic methods for the analysis of anatomical characters, there have remained some uncertainties about the composition, and therefore the monophyly, of the genus *Littorina*. The genus *Mainwaringia* consists of two species from India and Southeast Asia, and these are anatomically well known (Reid 1986*b*). Parsimony analysis resolved this genus near the base of the subfamily Littorininae, distant from the highly derived genus *Littorina* (Reid 1989). However, subjective weighting of certain characters shared by the two genera suggested the possibility of inclusion of *Mainwaringia* within *Littorina* (Reid 1989), with anomalous characters explained as adaptations to its unusual tropical and muddy habitat (Reid 1990*a*; Taylor & Reid 1990). The analysis of the 16S sequence has resolved this question, showing that *Mainwaringia* is in fact more distantly related to *Littorina* than are members of the genera *Littoraria* and *Nodilittorina*.

In earlier morphological cladistic analyses, *Littorina* has been defined as a monophyletic group by the two synapomorphies of paraspermatozoa without rod-pieces, and two consecutive spirals of the pallial oviduct

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(Reid 1989, 1990*a*). The revised morphological analysis presented here is consistent with this definition, although the addition of further outgroups is necessary to test these synapomorphies of the genus. Under this definition, the basal species *L. striata* is clearly included within *Littorina*. However, this species shares a number of plesiomorphic characters (numbers 1, 2, 9, 11, 12, 13; see tables 2 and 3) with the sister-genus *Nodilittorina*, in which it has sometimes been classified (Rosewater 1981; Bandel & Kadolsky 1982). Allozyme frequencies have also been used to examine the relationships of this problematic species, and in some analyses *L. striata* did cluster with other *Littorina* species rather than with a *Nodilittorina* species (Backeljau & Warmoes 1992). However, genetic distances were large, and this result was highly sensitive to both the measure of distance and the clustering method employed (Backeljau & Warmoes 1992; Backeljau *et al.* 1994), so that evidently allozyme data are unsuitable for resolution of this problem. In a preliminary analysis of 12S sequence data, using just two members of *Nodilittorina* as outgroup taxa, *L. striata* was shown to cluster with other *Littorina* species (Rumbak *et al.* 1994). A stronger test of the monophyly of *Littorina* requires inclusion of additional outgroups, as is possible using the 16S dataset. Surprisingly, the results show clustering of *L. striata* with the clade of three *Littoraria* species (a more distant outgroup than *Nodilittorina*, according to the morphological phylogeny of Reid 1989). However, as discussed above, this anomalous result can be explained by biases in base composition. Although the monophyly of *Littorina* has therefore not been unequivocally demonstrated by the molecular analyses, we conclude that there is not yet compelling reason to doubt it.

On the basis of morphological analysis, Reid (1989, 1990*a*) formalized the subgeneric division of *Littorina*, and this scheme is shown, superimposed on the consensus phylogeny, in figure 7. All the molecular analyses support the distant relationship of *L. striata* and *L. keenae*, both to each other and to the remaining *Littorina* species, so that their placement in two monotypic subgenera is appropriate. The classification of the remaining species into two subgenera, *Littorina* and *Neritrema*, recognizes their contrasting types of development, planktotrophy and nonplanktotrophy respectively. Although this is a useful biological distinction, with important genetic and evolutionary consequences, it remains unclear whether this division also reflects phylogenetic history. In the morphological analysis, the nominal subgenus appears as a paraphyletic group although, on the basis of the poorer resolution available from the molecular data, the possibility of its monophyly cannot be discounted.

A more interesting question concerns the possible monophyly of *Neritrema*, and hence the unique origin of nonplanktotrophic development within the genus. A molecular phylogeny of species of *Turritella* has recently been used to demonstrate the multiple origin of nonplanktotrophic development in that gastropod genus (Lieberman *et al.* 1993). In the morphological analysis of *Littorina*, the *Neritrema* clade is strongly supported by three unequivocal synapomorphies (characters 8, 9, 10; see tables 2 and 3, and figure 1). However, of these, one is the character of nonplanktotrophic development itself, and the other two relate to the production of a benthic spawn mass (an enlarged jelly gland for production of the spawn matrix, and simplification of the primitively biconvex egg capsules) and may be functionally correlated with developmental strategy. The molecular data are ambiguous on this question, for in only one analysis was there support for the monophyly of *Neritrema* (and then with the low bootstrap value of 54%), whereas two analyses contradicted it (table 5). As noted above, in the parsimony analysis of the combined molecular dataset (figure 4), the constraint of a monophyletic *Neritrema* did not alter the length of the most parsimonious trees found. The monophyly of *Neritrema* therefore remains unresolved. Even if paraphyly of the subgenus *Littorina*, and polyphyly of *Neritrema*, are demonstrated by future studies, it is already clear that the members of these two groups are more closely related than is suggested by their morphological diversity.

(*c*) *Rates of molecular evolution and ages of clades*

The estimation of average rates of molecular evolution is difficult and controversial, but is nevertheless potentially useful for evolutionary studies (Nei 1987; Gillespie 1991). The rates calculated here are presented only as coarse approximations, recognizing the problems inherent in their estimation. Rates may vary both within and between lineages although, within the genus *Littorina*, the assumption of uniformity of rates is not significantly violated. The ages of fossils are subject to some uncertainty, and furthermore provide only minimum estimates of ages of clades, which may lead to overestimation of rates. The pairwise distances between taxa of the same age vary widely, and have not been corrected to take account of multiple substitutions, which may result in underestimates of true rates of divergence. Despite these sources of error, the resulting linear regressions are all significant (figure 5), and can reasonably be used to estimate the ages of clades in the molecular phylogeny. Comparable rates for third position substitutions in the cytochrome-*b* gene are available for the muricid gastropods *Nucella* and *Plicopurpura* (Collins *et al.* 1996); for transitions these are 3–4% and 7.2% per Ma, respectively, and for transversions 0.42% and 2.4%. The corresponding rates for *Littorina* are 7.6% per Ma for transitions and 0.74% for transversions.

In view of the probable sources of error involved in the estimation of rates of molecular evolution, it is unsurprising that the corresponding estimates of the ages of clades vary quite widely (table 6). However, for the five points used for calibration of the rates, the overlaps between the molecular estimates of age and the independent estimates from palaeontological and geological evidence (figure 6) do give some confidence in the molecular ages of clades for which other evidence is not available. Estimates of the ages of divergence between pairs of sister-species can also be made from the genetic distance between them, as assessed from

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 7. *Estimates of ages of divergence of pairs of sister-species of* Littorina, *from genetic distance*

(Ages (Ma) based on Nei's (1972) genetic distance D measured from allozyme frequencies, and using the calibration of D by Nei (1987). References are to the measurement of D; only Zaslavskaya *et al.* (1992) and Tatarenkov (1995) have estimated ages in this way. Molecular ages estimated in the present study (from table 6) are given for comparison (range, and mean in parentheses).)

species pair	range of D	reference	age of divergence	molecular age
L. saxatilis, *L. arcana*	0.012–0.200	Knight *et al.* 1987	0.06–1.00	0–3.55 (1.73)
L. saxatilis, *L. compressa*	0.042–0.237	Ward, 1990	0.21–1.19	0–3.55 (1.73)
L. obtusata, *L. fabalis*	0.157–0.345	Tatarenkov, 1995	0.79–1.73	0–3.49 (1.32)
L. brevicula, *L. mandshurica*	0.569–0.573	Zaslavskaya *et al.* 1992	2.85–2.87	1.99–15.62 (5.84)
L. littorea, *L. squalida*	0.689	Zaslavskaya *et al.* 1992	3.45	3.28–10.07 (6.42)
L. scutulata, *L. plena*	0.276–0.477	Mastro *et al.* 1982, recalculated by Ward, 1990	1.38–2.39	2.48–18.63 (12.81)

allozyme studies (e.g. Nei 1987). In two studies of *Littorina* species, Nei's (1987) calibration of the genetic distance D has been employed in this way (Zaslavskaya *et al.* 1992; Tatarenkov 1995), according to which D equal to 1.0 corresponds to a divergence time of 5 Ma. Table 7 summarizes the available genetic distance data for pairs of likely sister-taxa, and applies Nei's calibration to estimate the corresponding ages. A different calibration (D of 1.0 corresponding to 7.7 Ma, derived from the pulmonate snail *Partula* by Johnson *et al.* 1986) was applied by Knight *et al.* (1987), in estimating the age of divergence between *L. saxatilis* and *L. arcana* as 0.34–0.55 Ma. Use of this alternative calibration would increase the ages in table 7 by 54%. Some of the estimates from allozymes in table 7 are close to those derived from the molecular calibrations, as in the case of the pair *L. obtusata* and *L. fabalis*. Others are not; the pair *L. scutulata* and *L. plena* is especially anomalous, because the molecular estimates (2.48, 11.94, 18.17, 18.63 Ma) are mostly much greater than the estimates from allozyme data (1.38 to 2.39 Ma).

As discussed earlier, the age of the genus *Littorina* is unlikely to be discovered from the fossil record, because of the lack of diagnostic synapomorphies of the shell. It can, however, be estimated by extrapolation of the rates of molecular evolution to the average sequence divergence between *Littorina* species and members of the probable sister-genus *Nodilittorina*. This gives values of 43 and 67 Ma, using 16S transversions and 12S transversions respectively. The possible *Littorina* species figured by Woods & Saul (1986) from the Palaeocene or Lower Eocene (approximately 55 Ma BP) might therefore date from a time close to the origin of the genus.

(*d*) *Historical biogeography*

With the availability of a dated phylogeny, geographical distributions of extant species, and the fossil record, it is possible to consider the historical biogeography and possible speciation mechanisms of the genus (Reid 1996). Here, we will consider two biogeographic hypotheses, relating to climatic vicariance in the northern Pacific and to the trans-Arctic interchange, that predict associations between the phylogenetic position of *Littorina* species and their geographical distribution (Reid 1990*b*). It has often been suggested that long-term climatic change during the Cenozoic has been an important cause of changing distribution patterns of shallow-water marine invertebrates in the northern Pacific, leading to vicariance and speciation (e.g. Golikov & Tzvetkova 1972; Vermeij 1989). Range shifts induced by climatic change may be particularly pronounced on north–south orientated coastlines. Here, for example, a cooling trend might cause a southward shift of the main population, leaving a northern subpopulation isolated in a warmer refugium, thus leading to genetic divergence and eventual speciation (Valentine & Jablonski 1983; Valentine 1984). Reid (1990*b*) suggested that under the influence of the general cooling trend during the later Cenozoic, this process could have led to younger species occupying successively more northerly ranges. In support, he used the earlier version of the morphological phylogeny of *Littorina*, in which *Mainwaringia* species, *L. mandshurica* plus *L. brevicula*, *L. squalida*, *L. aleutica* and *L. sitkana* were successive branches of a pectinate topology, and showed that although there was some overlap of their northern Pacific ranges, their mid-points were progressively further north. In this study, this precise correspondence between phylogeny (figure 7) and distribution (table 4) is not upheld.

Although the new results do not support the prediction of this speciation model of Valentine & Jablonski (1983), it is nevertheless likely that climatic fluctuations have had a strong influence on marine speciation processes in the northern Pacific. In the muricid gastropod *Nucella*, Collins *et al.* (1996) have identified a possible case of climatic vicariance caused by Pleistocene glaciation in the Aleutian Islands. In contrast, Ozawa & Okamoto (1993) have correlated Miocene speciation events in the Japanese trochid *Umbonium* with warm climatic intervals. From palaeontological evidence, the major polychotomy in the phylogeny of *Littorina* is estimated to have occurred about 12–15 Ma BP, and the corresponding molecular

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

estimate is 5.74–12.39 Ma BP (mean 9.60 Ma BP, table 6). The Middle Miocene was a time of unusually rapid climatic change, with climatic optima at about 15 and 11 Ma BP, separated by a cool interval (evidence from oxygen isotope analysis, Savin *et al.* 1981; microfossils, Barron & Baldauf 1990; fossil molluscs, Addicott 1969). The occurrence of fossils of *L. squalida* and *L. brevicula*, and of related Recent species, in the northwestern Pacific indicates that it was in this region that rapid cladogenesis took place. Climatic vicariance in this area of complex coastal topology may explain this burst of speciation, although further refinement of age estimation will be necessary before it can be connected with specific climatic events.

The trans-Arctic interchange was a major biogeographic event that took place during the Pliocene, 3.5–4 Ma BP, following the opening of the Bering Strait (Hopkins 1967). Many marine species were involved, including almost 300 molluscs, and in many cases speciation ensued following late Pliocene climatic cooling, which again isolated the two oceans. The interchange was markedly asymmetric, with the majority of species migrating from the Pacific to the Atlantic (Vermeij 1991). It is well known that *Littorina* took part in this interchange, and its older fossil record in the Pacific, and relatively recent appearance in the Atlantic, indicates that migration took place in the prevailing direction (Golikov & Tzvetkova 1972; Reid 1990*b*; Vermeij 1991). This leads to the prediction that the two clades of *Littorina* represented in the northern Atlantic (*L. littorea* and subgenus *Neritrema*) should have sister-taxa in the northern Pacific. The sister-species of the Atlantic *L. littorea* is the Pacific *L. squalida*, as suggested by the earlier studies and confirmed here. For the Atlantic *Neritrema* species, the earlier studies suggested that either *L. sitkana* (= *L. kurila*; Golikov & Tzvetkova 1972) or *L. subrotundata* (Reid 1990*a*) was the Pacific sister-taxon. However, this study indicates that this is likely to be either *L. aleutica* and *L. natica* (morphological analysis) or *L. natica* alone (16S analysis; see discussion above). New data on geographical distributions (Reid 1996) show that of all the Pacific *Littorina* species, only these three, *L. squalida*, *L. aleutica* and *L. natica*, commonly occur in the northern Bering Sea, where their immediate ancestors must have been in a position to migrate through the Bering Strait to the Atlantic.

The fossil evidence suggests that the ancestor of *L. littorea* may have reached the Atlantic very soon after the opening of the Bering Strait, for fossils morphologically similar to *L. squalida* occur in the Tjörnes Beds of Iceland at about 3.5 Ma BP, just before the main influx of Pacific species is recorded, and *L. littorea* first appears in the East Anglian Red Crag at about 2.4 Ma BP. For *Neritrema*, the first confirmed appearance in the Atlantic is also at Tjörnes, but at only 2 Ma BP(Reid 1996). If the hypothesis of vicariant speciation following trans-Arctic migration and subsequent isolation is correct, the estimated ages of separation of the two Atlantic clades from their Pacific sister-taxa should be less than the geological age of the opening of the Bering Strait (3.5–4 Ma, Hopkins 1967). As pointed out by Zaslavskaya *et al.* (1992), their estimate from allozyme data of the age of divergence between *L. squalida* and *L. littorea*, 3.45 Ma, is consistent with this hypothesis. However, the molecular estimates for this divergence are mostly greater (mean 6.42 Ma, table 6). There are several possible explanations for this discrepancy; speciation may have taken place in the Pacific before trans-Arctic migration occurred (considered unlikely, in view of the fossil evidence), the geological estimate of the age of the Strait might be questioned, or this lineage may have undergone unusually rapid molecular divergence. For *Neritrema*, the molecular estimates of separation between the Pacific and Atlantic clades are closer to the age of the Strait (mean 4.12 Ma, table 6).

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES