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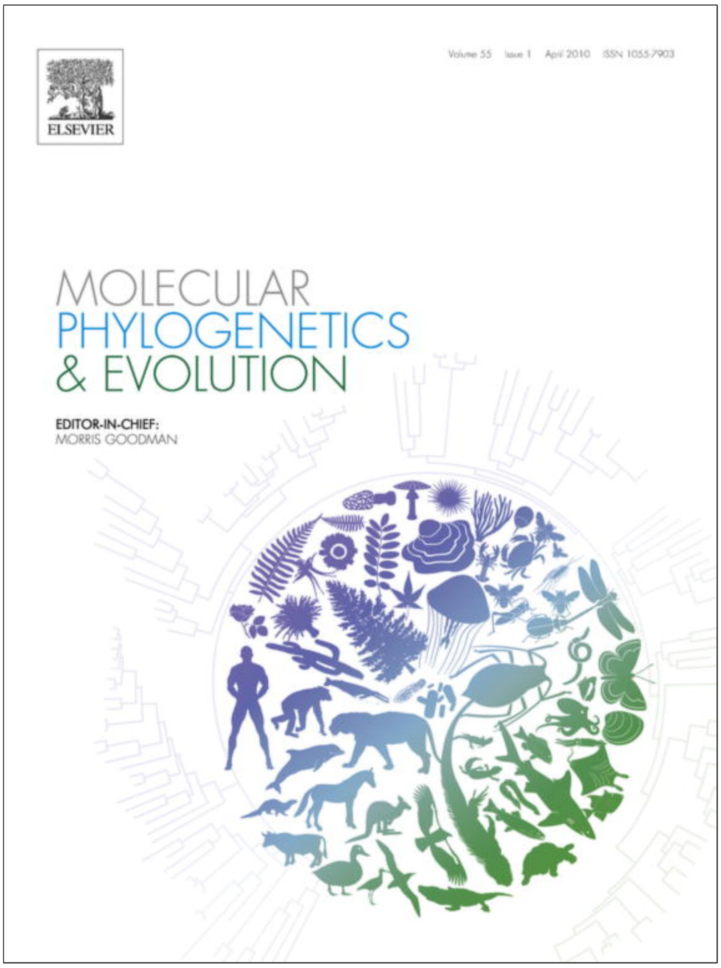
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Global diversification of mangrove fauna: a molecular phylogeny of *Littoraria* (Gastropoda: Littorinidae)

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ABSTRACT

The genus *Littoraria* is one of very few molluscan groups that are closely associated with mangroves. We document its global evolutionary radiation and compare biogeographic patterns with those of mangrove plants, based on phylogenetic and fossil evidence. Using sequences from three genes (nuclear 28S rRNA, mitochondrial 12S rRNA and COI) we reconstruct a phylogeny of 37 of the 39 living morphospecies. Six monophyletic subgenera are defined (*Bulimilittorina*, *Lamellilitorina*, *Littoraria*, *Palustorina*, *Protolittoraria*, *Littorinopsis*) and we synonymize *L. coccinea* and *L. glabrata*. A deep division between *Palustorina* from the Indo-West Pacific and *Littoraria* from the Atlantic and Eastern Pacific is estimated by a Bayesian relaxed-clock method to be of Middle Eocene to Palaeocene age (43.2–62.7 Ma), which far predates the Early Miocene (18 Ma) closure of the Tethyan Seaway; this, as in mangrove plants, may reflect vicariance by climatic cooling, rather than tectonic processes. The age of *Littoraria angulifera* in the Atlantic is, however, consistent with Early Miocene vicariance of a Tethyan ancestor. We infer that speciation events are mainly of Miocene or older age, and that diversification has not been driven by depletion of mangrove habitats during recent glacial intervals. Parsimonious reconstruction of ancestral habitats suggests that the genus has inhabited mangrove or wood substrates since its origin, while the rock-dwelling habit of the four members of *Protolittoraria* is derived. Three species span the Eastern Pacific Barrier, and one is amphi-Atlantic, consistent with a long larval phase of up to 10 weeks. Allopatric speciation is inferred, but usually with subsequent range overlap. Ovoviviparity (interpreted as an adaptation to life in mangroves) has arisen twice.

1. Introduction

Mangrove forests dominate about one quarter of tropical coastlines (Spalding et al., 1997). However, they are among the "most threatened major environments on earth", global loss through human activity having been estimated at 35% (Valiela et al., 2001), while some of the remaining forests are vulnerable to rising sea-levels (Ellison, 1994). These marine forests support a distinctive fauna, and the macroinvertebrates are dominated by molluscs and crabs (Macnae, 1968). For some animals, particularly those inhabiting only the seaward fringes of mangroves, the trees simply provide a hard substrate on otherwise sedimentary shores. However, the forest as a whole presents a unique and challenging habitat, characterized by shade from the leaf canopy, a three-dimensional structure of trunks and roots, a rich supply of decaying plant material, acidic and anoxic soil, variable salinity and intermittent tidal inundation, and strong predation by crabs and vertebrates. There is a small group of animals, mainly molluscs, that show an obligate association with the mangrove habitat (Plaziat et al., 2001) and these display a corresponding suite of adaptations (Vermeij, 1973; Reid, 1992).

The existence of species and groups of animals that are strongly associated with mangroves raises at least three evolutionary questions. Did the shift to the mangrove habitat initiate an adaptive radiation? Do the animals show patterns of global biogeographic history that are similar to those of the mangrove plants? Does the nature of the mangrove environment dictate the tempo and mode of speciation of its inhabitants? So far, some of these questions have been considered in a molecular phylogenetic context only for one family of gastropod molluscs, the Potamididae (Reid et al., 2008) and two families of grapsoid crabs (Gillikin and Schubart, 2004; Fratini et al., 2005; Ragionieri et al., 2009), although a few mangrove-associated species have also been included within larger radiations of gastropods on rocky shores (Littorinidae, Williams et al., 2003; *Nerita*, Frey and Vermeij, 2008).

Evidence for an adaptive radiation in the mangrove habitat comes from the age and location of fossils, and from phylogenetic reconstruction of ancestral habitats. The oldest fossil evidence for a modern mangrove genus (the palm *Nypa*) is from the Late Cretaceous, but most groups arose during the Palaeocene and diverse mangrove forests were established by the Early Eocene (Ellison

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et al., 1999; Plaziat et al., 2001). For the Potamididae, both their fossil record and ancestral habitat are consistent with the hypothesis of adaptive radiation of the extant genera in the new mangrove habitat following its appearance in the early Tertiary (Reid et al., 2008). For mangrove littorinids the fossil record is equally old, but there is conflicting phylogenetic evidence for an ancestral habitat on either rocky shores or mangroves (Reid, 1999b; Williams et al., 2003).

The mangrove plants consist of about 70 species worldwide, comprising trees, shrubs and ferns that belong to 21 family-level lineages each independently derived from non-mangrove ancestors (Ellison et al., 1999; Ricklefs et al., 2006). There has been much interest in the historical biogeography of mangroves, in particular attempting to explain why their diversity is strongly focused near the center of the Indo-West Pacific (IWP) region. (This has been called the 'mangrove biodiversity anomaly', e.g. Ellison et al., 1999; Ricklefs et al., 2006, although it is not anomalous in the context of the similar pattern displayed by many marine animal groups.) The limited fossil record of mangroves supports an early Tertiary origin in the Tethyan Realm, followed by pantropical distribution, disjunction when the Tethys was closed in the Miocene, and partial extinction in the Atlantic (McCoy and Heck, 1976; Ellison et al., 1999; Plaziat et al., 2001). Molecular phylogenetic evidence suggests that most mangrove lineages originated in the Old World (Ricklefs et al., 2006) and diversified within the IWP (Duke et al., 1998; Liao et al., 2007), although there have yet been no molecular phylogenetic studies at species-level of entire mangrove clades, even for the more species-rich and widespread groups such as *Avicennia* and the Rhizophoraceae. Clades of mangrove-associated animals that originated at the same time as mangroves are expected to show similar patterns (Ellison et al., 1999). So far there is only limited evidence of this in the Potamididae, which show maximum diversity in the central IWP, fossil evidence of extinction in the Atlantic, and one case of a sister-relationship between clades in the IWP and the Americas, implying possible vicariance (Reid et al., 2008).

Molecular phylogenetic studies are producing a wealth of new data on the geographical pattern and age of speciation events in the marine realm, although large and well-sampled species-level phylogenies are not yet available for mangrove molluscs. As new phylogenies accumulate, it is becoming clear that while the prevailing mode of speciation is of the classical large-scale allopatric kind, the necessary geographical disruption can be achieved by a variety of diverse mechanisms, involving vicariance by tectonic, climatic or oceanographic means, or dispersal across pre-existing barriers (Bellwood and Wainwright, 2002; Williams and Reid, 2004; Read et al., 2006; Floeter et al., 2008; Frey and Vermeij, 2008). Although the sea-level changes associated with Pleistocene glaciations were formerly considered to have driven speciation events, most estimates of sister-species divergences are older, from early Pliocene to Miocene age (Bellwood and Meyer, 2009). So far, global phylogenetic studies of species-level diversification in the tropics, with fossil calibration, have been based largely on organisms from coral reefs (Duda and Kohn, 2005; Read et al., 2006; Williams and Duda, 2008) or rocky shores (Williams and Reid, 2004; Frey and Vermeij, 2008), and it is unclear whether the same conclusions hold for other habitats. Potentially, there are several reasons why speciation processes in the mangrove habitat might display differences. The mangrove habitat is highly sensitive to sea-level change, being greatly reduced in global area during low sea-level stands (Woodroffe and Grindrod, 1991; Sun et al., 2000). The corresponding local extinctions and vicariance of associated fauna might therefore have been more severe than on reefs and rocky shores, promoting speciation in mangrove habitats during the Pleistocene glaciation cycles. Through a different mechanism, during the emergence of the Isthmus of Panama the inhabitants of shallow-sedimentary and mangrove habitats were among the last to be divided during the gradual shoaling and uplift of the barrier, again leading to relatively younger ages of sister-species in these habitats (Knowlton and Weigt, 1998; Hurt et al., 2008). Since they flourish in sedimentary environments with high nutrient status, mangroves are most well developed on continental margins, and are depauperate or absent on oceanic islands. This too may influence speciation processes, because connectivity of populations is greater on continuous coastlines and allopatry, while more difficult to achieve, is also more likely to be transient, leading to contrasting patterns on continental margins and oceanic archipelagos (Hellberg, 1998; Williams and Reid, 2004). Nevertheless, mangrove communities can also be found under oceanic conditions of low nutrient status (Woodroffe and Grindrod, 1991), and the molluscan communities associated with continental and oceanic mangroves differ in composition (Reid, 1986a). This raises the possibility of parapatric ecological speciation between continental and oceanic habitats, as recently investigated in other marine groups (Rocha et al., 2005; Reid et al., 2006). For all these reasons, the study of speciation in mangrove-associated fauna should provide a new perspective on diversification in the marine realm.

The molluscs that inhabit mangrove forests are not diverse. In a worldwide survey of gastropods associated with mangrove biotopes, Cantera et al. (1983) listed 277 species. This, however, is misleading, because more than half are from adjacent mudflats or rocky shores, or are micromolluscs from brackish landward areas, and have no obligate association with mangroves. The true mangrove associates are mostly marine snails, found predominantly or exclusively in this environment, variously dependent on the trees for substrate, food, shade or protection from predators (Vermeij, 1973; Plaziat, 1984; Reid, 1986a; Plaziat et al., 2001; Lozouet and Plaziat, 2008; Reid et al., 2008). These mangrove gastropods include less than 20 species of *Nerita*, *Cerithium* and Muricidae, and an indeterminate number of the more diverse but poorly studied Ellobiidae. The most conspicuous and abundant of mangrove gastropods belong to the families Potamididae (29 species, mostly associated with mangroves, Reid et al., 2008) and Littorinidae.

Our study group is the littorinid genus *Littoraria*. Most other members of the family Littorinidae are inhabitants of rocky shores, and several have already been the subject of studies of global biogeography and speciation (e.g. *Littorina*, Reid, 1996; Reid et al., 1996; *Austrolittorina*, Williams et al., 2003; *Echinolittorina*, Williams and Reid, 2004; Williams and Duda, 2008). Only two littorinid clades have a strong association with mangroves: *Mainwaringia* with just two species, both in Southeast Asia (Reid, 1986b), and *Littoraria*. The morphological taxonomy, habitat and geographical distributions of *Littoraria* species are well known (Reid, 1986a; Reid, 1999a,b, 2001; Stuckey and Reid, 2002). All of the 39 recognized species are of tropical or subtropical distribution, and the great majority occur mainly on mangrove trees or other halophytic plants, in salt marshes and on driftwood, although at least five species are found most frequently on rocky shores and others can occasionally occupy rocks in sheltered inlets. Adaptations to this habitat shown by *Littoraria* species include shell architecture (Reid, 1992), shell color polymorphism (Reid, 1987; McKillup and McKillup, 2008), ovoviviparous development (Reid, 1989), activity rhythms (Ohgaki, 1992; Lee and Williams, 2002), plasticity of radular teeth (Reid and Mak, 1999) and other dietary innovations (Lee et al., 2001; Silliman and Newell, 2003). Diversity is highest in the IWP with 28 species, while six inhabit the eastern Pacific and a further six the Atlantic Ocean (Table 1). Development is planktotrophic in all but one of the species, with a larval duration estimated at 3–10 weeks (Reid, 1986a, 1989). The fossil record of littorinids is scant, but that of *Littoraria* is richer than most and has been briefly reviewed (Reid, 1999b).

Table 1
Species of *Littoraria* used in this study, with geographical ranges and localities of samples. All material newly sequenced except for four samples as indicated (from Williams et al., 2003). All voucher material is deposited in the Natural History Museum, London. Taxonomy and ranges from Reid (1986a, 1999a, 2001) and Stuckey and Reid (2002).

Species	Range	Sample location and code
L. aberrans (Philippi, 1846)	W Panama	1, 2: Playa Panama, 2 km N Rio Grande, Nicoya Peninsula, Costa Rica
L. albicans (Metcalfe, 1852)	Borneo	1: Bako, Sarawak
		2: Santubong, Sarawak
L. angulifera (Lamarck, 1822)	W and E tropical Atlantic O.	1: Fort Charlotte, Lucea, Jamaica
		2: Caraneia, near Iguape, Sao Paulo State, Brazil
		3: Sine-Saloum Delta, near Djifere, Senegal
		4: Gao Lagoon, East Tema, Ghana
L. ardouiniana (Heude, 1885)	NW Pacific O.	1: Bali, Tamshui R., Taiwan
		2: Lo R., Nha Trang, Vietnam
L. articulata (Philippi, 1846)	Central IWP	1: Sarimbun, Singapore
		2: Knobby Head, Cockle Bay, Magnetic I., Queensland, Australia
		3: Karratha Back Beach, WA, Australia
		4: Xiamen, China
L. bengalensis Reid, 2001	NE Indian O.	1: Kuah, Langkawi, Malaysia
		2: Hare I., Gulf of Mannar, India
L. carinifera (Menke, 1830)	Central IWP	1: Tanjung Rhu, Langkawi, Malaysia
		2: Lembar, Lombok, Indonesia
		3: Gaya I., Kota Kinabalu, Sabah, Malaysia
L. cingulata cingulata (Philippi, 1846)	NW Australia	1: Crab Creek, 30 km S Broome, WA, Australia
		2: Karratha Back Beach, WA, Australia
L. cingulata pristissini Reid, 1986	Shark Bay, W Australia	1: 1 km S Monkey Mia, Shark Bay, WA, Australia
		2: Little Lagoon, Denham, Shark Bay, WA, Australia
L. cingulifera (Dunker, 1845)	W Africa	1, 2: Ankobra R., 70 km W Takoradi, Ghana
L. coccinea (Gmelin, 1791)	Pacific O.	1: Namaqaqua, Viti Levu, Fiji
		2: Ishigaki I., Japan
L. conica (Philippi, 1846)	Central IWP	1, 2: Lim Chu Keng, Singapore
L. filosa (Sowerby, 1832)	N Australia	1: Weld Street, Broome, WA, Australia
		2: Cockle Bay, Magnetic I., Queensland, Australia
L. flava (King & Broderip, 1832)	W Atlantic	1: Caraneia, near Iguape, Sao Paulo State, Brazil
		2: Bara Cunha, Curimatau Estuary, 60 km S Natal, Brazil
L. glabrata (Philippi, 1846)	Indian O.	1: Mangapwani, Zanzibar, Tanzania
		2: Weligama, Sri Lanka
L. ianthostoma Stuckey & Reid, 2002	Joseph Bonaparte Gulf, N Australia	1: Forsyth Creek, Joseph Bonaparte Gulf, NT, Australia
L. intermedia (Philippi, 1846)	IWP	1: Lilipuna Dock, Kaneohe Bay, Oahu, Hawaii.
		2: Honjo R., Kushima, Miyazaki, Kyushu, Japan
		3: Magellan Bay, Punta Engano, Mactan I., Cebu I., Philippines
		4: SE Pemba Bay, Cabo Delgado Prov., Mozambique
		5: Nabq, Gulf of Aqaba, Egypt
		6: Rowes Bay, Townsville, Queensland, Australia
		7: Hare I., Gulf of Mannar, India
L. irrorata (Say, 1822)	SE USA	1, 2: Dauphin I., Alabama, USA
L. lutea (Philippi, 1847)	W Pacific O.	1, 2: Magellan Bay, Punta Engano, Mactan I., Cebu I., Philippines
L. luteola (Quoy & Gaimard, 1833)	E Australia	1: Lake Perimbula, NSW, Australia
		2: Bonna Point, Kurnell Peninsula, Botany Bay, NSW, Australia
L. mauritiana (Lamarck, 1822)	W Indian O.	1: Libanona Beach, Taolagnaro, Madagascar
		2: Grande Riviere Noire, Mauritius
L. melanostoma (Gray, 1839)	Central IWP	1: Sarimbun, Singapore
		2: Tuan Chau I., Ha Long Bay, Vietnam
L. nebulosa (Lamarck, 1822)	W Atlantic O.	1: 20 km S Campeche, Mexico
		2: Playa Villarica, Actopan, Veracruz, Mexico
L. pallescens (Philippi, 1846)	IWP	1: Makiya, Okinawa, Japan
		2: Ibo I., Cabo Delgado Prov., Mozambique
		3: Gaya I., Kota Kinabalu, Sabah, Malaysia
L. philippiana (Reeve, 1857)	NE Australia	1: Port Douglas, Queensland, Australia
		2: Rowes Bay, Townsville, Queensland, Australia
L. pintado pintado (Wood, 1828)	IWP	1: Ishigaki, Japan (from Williams et al., 2003)
		2: Kaena Point, Oahu, Hawaiian Is
		3: Libanona Beach, Taolagnaro, Madagascar
		4: E Rodrigues I.
L. pintado pullata (Carpenter, 1864)	E Pacific O.	1: Cabo San Lucas, Baja California, Mexico
		2: Wafer Bay, Isla del Coco, Costa Rica
L. rosewateri Reid, 1999	E Pacific O.	1: Opposite Isla Damas, Quepos, Costa Rica
		2: Muisne, Esmeraldas Prov., Ecuador
L. scabra (Linnaeus, 1758)	IWP	1: Hare I., Gulf of Mannar, India (from Williams et al., 2003)
		2: Ibo I., Cabo Delgado Prov., Mozambique
		3: Naha, Okinawa, Japan
		4: Dili Bay, East Timor
		5: Pioneer Bay, Orpheus I., Queensland, Australia
		6: Vatia Point, Viti Levu, Fiji
L. sinensis (Philippi, 1847)	NW Pacific O.	1: Chiku, 15 km N Tainan, Taiwan
		2: Honjo R., Kushima, Miyazaki, Kyushu, Japan
L. strigata (Philippi, 1846)	Central IWP	1: Pelangi Beach, Langkawi, Malaysia
		2: Kao Sam Muk, Bangsaen, Tumbon Saensuk, Ampur Moeng, Chonburi, Thailand
		3: Tanjung Kait Beach, NW Jakarta, Java

(*continued on next page*)

Table 1 (*continued*)

Species	Range	Sample location and code
L. subvittata Reid, 1986	W Indian O.	1: Ibo I., Cabo Delgado Prov., Mozambique 2: Toliara, Madagascar
L. sulculosa (Philippi, 1846)	NW Australia	1: Hearson's Cove, Dampier, WA, Australia 2: Karratha Back Beach, WA, Australia
L. tessellata (Philippi, 1847)	Caribbean Sea	1: Boca del Rio Brimbo, Cienfuegos, Cuba 2: Bocas del Toro, Panama
L. undulata (Gray, 1839)	IWP	1: Borongan, Samar I., Philippines (from Williams et al., 2003) 2: Kappad Beach, 16 km N Kozhikode, India 3: Dar es Salaam, Tanzania 4: Loomis Beach, Lizard I., Queensland, Australia 5: Chialoshui, 10 km E Hengchun, Taiwan
L. varia (Sowerby, 1832)	E Pacific O.	1: Playa Panama, 2 km N Rio Grande, Nicoya Peninsula, Costa Rica 2: Estero Salado, Guayaquil, Ecuador
L. variegata (Souleyet, 1852)	E Pacific O.	1: San Lorenzo, Esmeraldas Prov., Ecuador 2: Topolobampo, 20 km W Los Mochis, Mexico
L. vespacea Reid, 1986	Central IWP	1: Sungi Buloh Wetland Reserve, Singapore 2: Santubong, Sarawak
L. zebra (Donovan, 1825)	E Pacific O.	1: San Lorenzo, Esmeraldas Prov., Ecuador (from Williams et al., 2003) 2: Playa Pochote, Nicoya Peninsula, Costa Rica

There have been several attempts to reconstruct the phylogeny of *Littoraria* species. Cladistic analyses of morphological characters have included all the known species and are the basis for the present subgeneric classification (Reid, 1986a, 1999b). Allozyme frequencies in 11 mainly Australian species have been analysed by phenetic and phylogenetic methods, and gave some support to subgeneric groupings (Inness-Campbell et al., 2003). Most recently, one representative of each of the four main subgenera was included in a molecular phylogenetic analysis (based on two nuclear and two mitochondrial genes) of the subfamily Littorininae, and yielded a new topology (Williams et al., 2003).

Here we report a molecular phylogenetic analysis of all but one of the living *Littoraria* species. This genus now joins the few species-rich molluscan clades of worldwide tropical distribution for which a near-complete phylogeny is available (Cypraeidae: Meyer, 2003, 2004; *Echinolittorina*: Williams and Reid, 2004; Williams and Duda, 2008; *Conus*: Duda and Kohn, 2005; *Nerita*: Frey and Vermeij, 2008). The phylogenetic hypothesis provides a test of the existing subgeneric and species-level classification based on morphological characters. We assess the biogeography and adaptive radiation of the genus in the light of its phylogeny, fossil record and the habitats of its living species, and of the history of mangroves. Based on known geographical distributions and fossil calibration of divergences, we attempt to reconstruct the age and geography of speciation events, comparing these with patterns of diversification on rocky shores and reefs. Based on geographical ranges and the phylogenetic pattern of reproductive mode, we make inferences about the evolution of life history and the likely length of larval life.

2. Material and methods

2.1. Sampling, identification, species status and geographical distribution

The ingroup consisted of 37 nominal species and two subspecies of the genus *Littoraria* (Table 1). Species-level taxonomy followed that of Reid (1986a, 1999a, 2001) and Stuckey and Reid (2002), using diagnostic morphological differences in shells and genital anatomy for identification. Only two of the 39 recognized species were not sampled: *L. delicatula* (Nevill, 1885) from the Bay of Bengal, and *L. flammea* (Philippi, 1847) from China (believed to be extinct; Carlton, 1993). In order to evaluate the status of the morphospecies as monophyletic units, in most cases at least two specimens were sampled from widely separated localities. For the rare *L. ianthostoma*, restricted to Joseph Bonaparte Gulf in northwestern Australia, only a single specimen was available. For the most geographically widespread taxa and those in which regional variation in shell morphology hinted at possible cryptic species, up to seven individuals were sampled. Subgeneric nomenclature followed that of Reid (1999b), but with reassignment of two species (see Fig. 2 and Section 4); the subgenera serve as a useful means of referring to the principal clades. Detailed locality records and distribution maps were available for all species (Bequaert, 1943; Rosewater, 1970; Reid, 1986a, 1999a, 2001; Reid and Kaiser, 2001; Stuckey and Reid, 2002; Reid, unpublished). The monophyly and sister-relationship of the genus *Littoraria* are not in doubt (Williams et al., 2003) and therefore only the sole member of the monotypic sister genus *Cenchritis* was used as outgroup. Voucher material is deposited in the Natural History Museum (London) (BMNH).

2.2. DNA extraction, amplification and sequencing

DNA was extracted from mantle or foot tissue of ethanol-preserved material using the CTAB extraction method of Williams et al. (2003). Tissue was blotted dry and soaked for up to 1 h in 0.01 TE buffer to remove ethanol. Tissue was placed in a microfuge tube containing 300 μl of CTAB extraction buffer pre-heated to 56 °C (100 mM Tris HCl, pH 8.0, 1.4 M NaCl [incorrectly reported as 1.4 mM], 20 mM EDTA, 2% w/v CTAB, 2% w/v PVP 40,000 MW, with 0.2% β-mercaptoethanol added just before use), then macerated and incubated overnight at 56 °C with 20 μl of proteinase K (20 mg/ml). The tissue extract was then extracted at least twice with chloroform, precipitated with two volumes of ethanol in the presence of sodium acetate, and finally resuspended in 0.01 TE buffer.

Portions of three genes were amplified and sequenced: the nuclear 28S rRNA gene (approximately 1500 bp) and the mitochondrial 12S rRNA (approximately 410 bp) and cytochrome-*c* oxidase I (COI) genes (709 bp). These three gene regions have been shown to evolve at suitable rates for the reconstruction of species-level phylogenies of littorinid gastropods (Williams et al., 2003; Williams and Reid, 2004). Amplification and sequencing methods used were those described by Williams et al. (2003) with 28S sequencing primers from Williams and Ozawa (2006). Sequence reactions were performed directly on purified PCR products using a BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) and

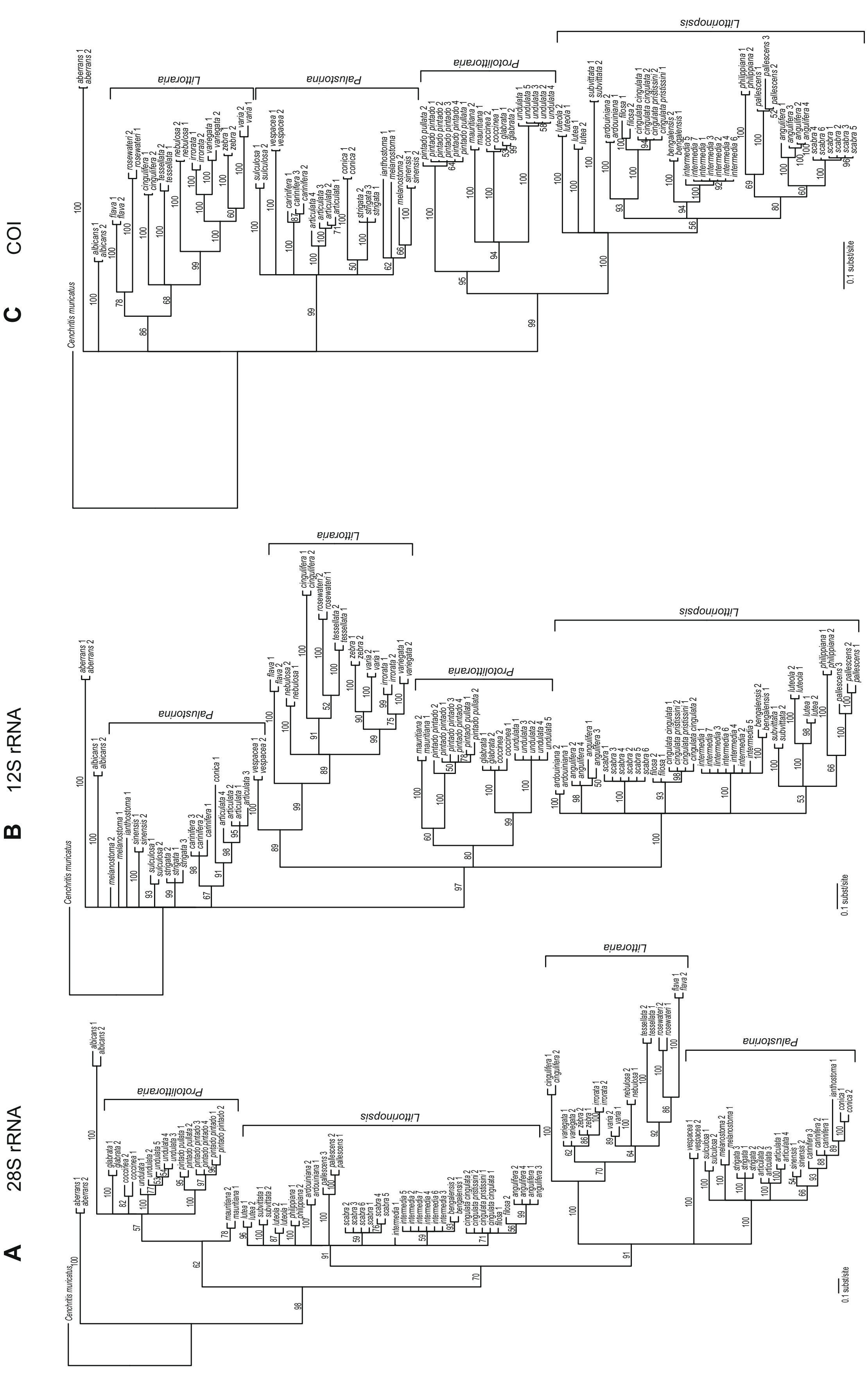


Fig. 1. Molecular phylogenies of *Littoraria* species produced by Bayesian analysis of datasets from individual genes, the nuclear 28S rRNA (A) and mitochondrial 12S rRNA (B) and COI (C), using *Cenchritis muricatus* as outgroup. The taxonomy follows morphospecies concepts of Reid (1986a, 1999a, 2001) and Stuckey and Reid (2002). Sample localities are listed in Table 1. Support values are Bayesian posterior probabilities (%). Square brackets indicate the monophyletic subgeneric groupings supported by the combined-gene analysis (Fig. 2). Two species are classified in monotypic subgenera: *L.* (*Bulimilittorina*) *aberrans* and *L.* (*Lamellilittorina*) *albicans*.

run on a 3730 DNA Analyser automated capillary sequencer (Applied Biosystems).

Sequences were verified by forward and reverse comparisons. Gene sequences for the outgroup and for single individuals of four *Littoraria* species (Table 1) were taken from Williams et al. (2003). All new sequences have been deposited in GenBank (accession numbers: 28S FN556254–556347; 12S FN556468–556560; COI FN557067–557160).

2.3. Sequence analysis and phylogeny reconstruction

Sequences were edited using Sequencher (v 4.8, Gene Codes Corporation, Ann Arbor, Michigan). Sequences of ribosomal genes were aligned using Clustal X (Thompson et al., 1994, 1997) ('delay divergent sequence' = 95%, gap-opening penalty = 20, gap-extension penalty = 5) with minor adjustments made by eye in MacClade (v 4.06 OSX; Maddison and Maddison, 2003). Poorly aligned sites were identified using Gblocks (v 0.19b, Castresana, 2000) and removed from analyses with parameters set as by Williams and Ozawa (2006). After removal of these ambiguous blocks of data a total of 1458 bp of sequence from 28S rRNA (98% of 1476 bp) and 359 bp of sequence from 12S rRNA (94% of 378 bp) remained for analysis.

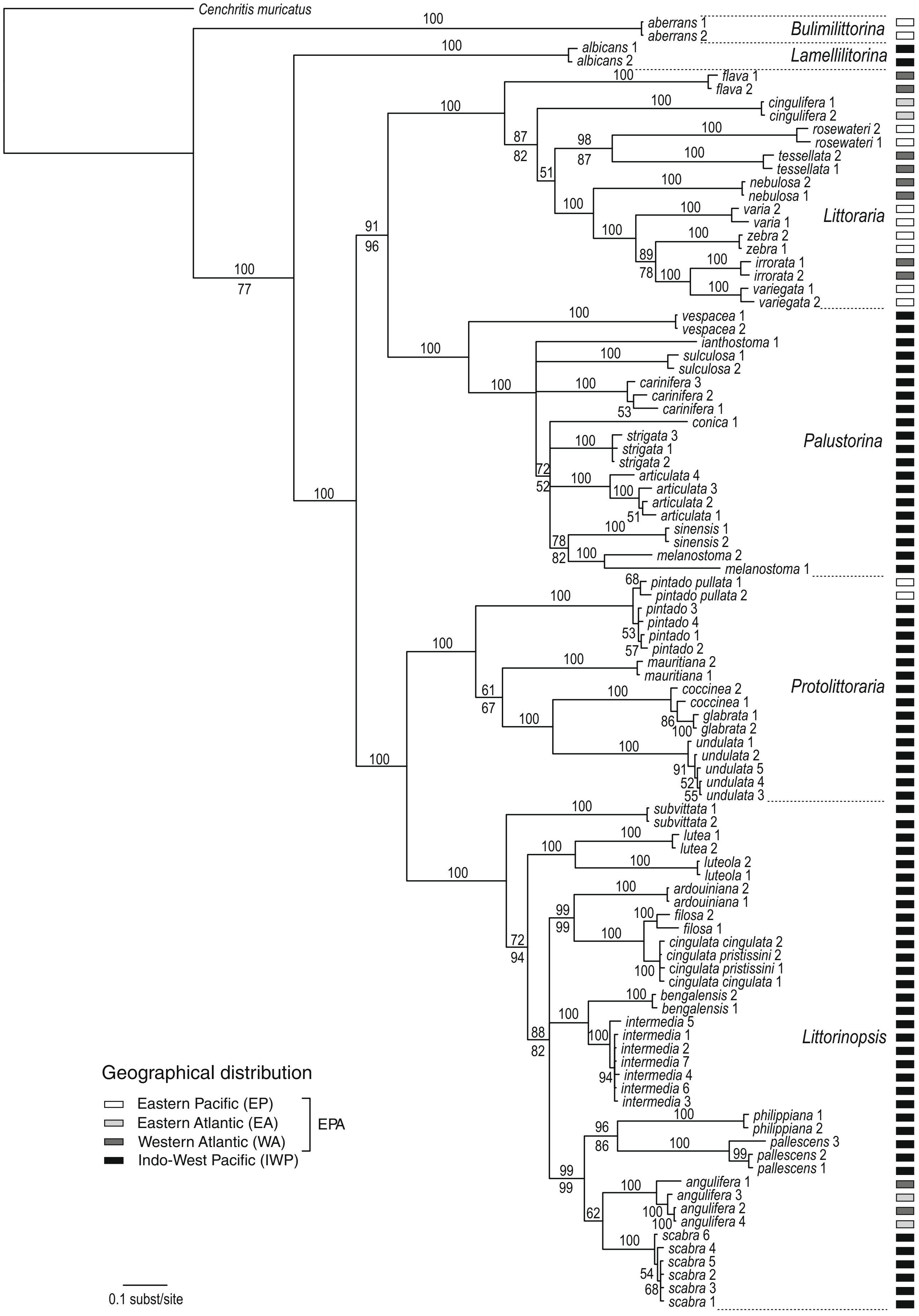
Phylogenies were constructed using Bayesian methods implemented by MrBayes (v 3.1.2, Huelsenbeck and Ronquist, 2001). Trees were obtained for each gene-sequence dataset (Fig. 1) and for a combined dataset that included all individuals for which all three genes were available (Fig. 2). Models to be used in Bayesian analyses were determined with MrModelTest (v 2.1, J. Nylander, www.ebc.uu.se/systzoo/staff/nylander.html). The COI dataset was tested additionally to see whether variation across codon positions would result in an improved likelihood. The best model for all datasets was determined to be GTR+G+I using the hierarchical likelihood ratio tests. These models were used in all MrBayes analyses, with all parameters free to vary. In the combined analyses, variation was partitioned among genes and gene-specific model parameters were used (with all parameters free to vary independently within each partition). In addition each gene was allowed to evolve at a different rate. The analysis for each data set was run for 7,500,000 generations, with a sampling frequency of 1000. The first 3501 trees were discarded, so that 4000 trees were accepted for each run after likelihood values had reached a plateau. The datasets were analysed in two independent runs and the final tree was computed from the combination of accepted trees from each run (a total of 8000 trees). Convergence between the two runs was tested by examining the potential scale reduction factors (PSRF) produced by the 'sump' command in MrBayes. All runs resulted in PSRF values of one for all parameters. Support for nodes was determined using posterior probabilities (PP, calculated by MrBayes).

2.4. Divergence times

Divergence times between clades were estimated with BEAST (v 1.4.8, Drummond and Rambaut, 2007). This is a Bayesian Markov chain Monte Carlo method that incorporates a relaxed molecular-clock model and multiple calibration points. Rates are uncorrelated across the tree, drawn independently from a parametric distribution (Drummond et al., 2006; Drummond and Rambaut, 2007; Ho, 2007). We used the relaxed uncorrelated lognormal clock model, in which the rate on each branch is independently drawn from a lognormal distribution (Drummond et al., 2006) with a birth–death process. For the COI gene the HKY+Γ+I substitution model was used after initial analyses showed that this was better than GTR+Γ+I (based on log Bayes Factors), and for the 12S and 28S rRNA genes GTR+Γ+I. The combined dataset was partitioned among genes, gene-specific models were used, and all parameters were unlinked. Analyses were undertaken with 50×10^6 generations and a sampling frequency of 1000. Convergence was assessed using Tracer v 1.4 (Rambaut and Drummond, 2007), with a discarded burn-in period of 5000 trees for the datasets of individual genes and 45,000 trees for the combined dataset. On the basis of preliminary analyses Jeffries priors were changed to lognormal priors for very low GTR base substitutions ($\leqslant 10^{-3}$) (12S: CG, GT) as used by Williams and Duda (2008) on the basis of a significantly higher Bayes Factor and improved ESS values ($\gg$200).

The sparse fossil record of *Littoraria* has been reviewed by Reid (1999b). This was used as a source of potential calibration points, but with revised ages (according to Ogg et al., 2008, and references given below). All calibration points were assigned a range of dates, with a minimum based on fossil evidence and a lognormal prior. The earliest known probable member of *Littoraria* is *L. densestriata* (Deshayes, 1861) from the Ypresian stage of the Early Eocene (48.6–55.8 Ma) of France. This species cannot be assigned to any of the extant subgenera and was used to estimate the age of the crown-group node for the genus (median: 48.6 Ma; std dev: 0.1; zero offset: 7.37 Myr; interval: 48.6–64.7 Ma). *Littoraria subangulata* (Deshayes, 1861) resembles living members of the subgenus *Littorinopsis*. This species, from the Bartonian stage of the Middle Eocene (37.2–40.4 Ma) of France, was therefore placed at the crown-group node of *Littorinopsis* (median: 37.2 Ma; std dev: 0.09; zero offset: 5.12 Myr; interval: 37.2–48.3 Ma). A specimen from the uppermost Culebra Formation of the Lower Miocene (19.1–19.8 Ma; Kirby et al., 2008) of Panama resembles the Recent species *L. (Littorinopsis) angulifera* (see Woodring, 1957) and this provided an estimate for the divergence between *L. angulifera* and *L. scabra* (median: 19.1 Ma; std dev: 0.1; zero offset: 2.9 Myr; interval: 19.1–25.4 Ma). There are several records for potential dating of the lineage of *L. irrorata* in the southeastern USA. Two probably synonymous fossil taxa from Florida resemble *L. irrorata*; these are *L. sheaferi* (Olsson, 1967), described from the Upper Pinecrest beds of the Pliocene (2.0–2.5 Ma; Allmon et al., 1996; Vermeij, 2005) and *L. caloosahatcheensis* (Petuch, 1991) from the Caloosahatchee Formation (1.8–2.5 Ma; Allmon et al., 1996). There are many records of *L. irrorata* itself from the Pliocene Yorktown Formation (Gardner, 1948), dated at 3.0–4.0 Ma (Dowsett and Wiggs, 1992). It has also been mentioned from the Late Miocene Choptank Formation (Gardner, 1948; also Bequaert, 1943: "Upper Miocene"), dated at 8.2–10.5 Ma (Kidwell, 1989), but no figure or specimen has been seen. Reflecting this uncertainty, the separation of *L. irrorata* from its sister taxon *L. variegata* was estimated at 3.0–10.5 Ma (median: 3.1 Ma; std dev: 1; zero offset: 2.50 Myr; interval: 3.1–18.6 Ma).

Fig. 2. Molecular phylogeny of *Littoraria* species produced by Bayesian analysis of combined dataset from concatenated sequences of the nuclear 28S rRNA and mitochondrial 12S rRNA and COI genes, using *Cenchritis muricatus* as outgroup. Sample localities and geographical distributions are listed in Table 1. Support values are Bayesian posterior probabilities (%); those above branches were generated by MrBayes analysis, and those below are the values for the corresponding clades generated by BEAST analysis (where both analyses gave 100% support, the BEAST probability is omitted). The species-level taxonomy follows traditional morphospecies concepts (Reid, 1986a, 1999a, 2001; Stuckey and Reid, 2002; Table 1), but it is recommended that *L. glabrata* should be reduced to a subspecies of *L. coccinea* (see Section 4). Subgeneric taxonomy follows that of Reid (1986a, 1989, 1999b), except that *L. ianthostoma* and *L. vespacea* are added to the subgenus *Palustorina*.


Cenchritis muricatus
aberrans 1
aberrans 2
Bulimilittorina
albicans 1
albicans 2
Lamellilitorina
flava 1
flava 2
cingulifera 1
cingulifera 2
rosewateri 2
rosewateri 1
tessellata 2
tessellata 1
nebulosa 2
nebulosa 1
varia 2
varia 1
Littoraria
zebra 2
zebra 1
irrorata 1
irrorata 2
variegata 1
variegata 2
vespacea 1
vespacea 2
ianthostoma 1
sulculosa 1
sulculosa 2
carinifera 3
carinifera 2
carinifera 1
conica 1
Palustorina
strigata 3
strigata 1
strigata 2
articulata 4
articulata 3
articulata 2
articulata 1
sinensis 1
sinensis 2
melanostoma 2
melanostoma 1
pintado pullata 1
pintado pullata 2
pintado 3
pintado 4
pintado 1
pintado 2
mauritiana 2
mauritiana 1
Protolittoraria
coccinea 2
coccinea 1
glabrata 1
glabrata 2
undulata 1
undulata 2
undulata 5
undulata 4
undulata 3
subvittata 1
subvittata 2
lutea 1
lutea 2
luteola 2
luteola 1
ardouiniana 2
ardouiniana 1
filosa 2
filosa 1
cingulata cingulata 2
cingulata pristissini 2
cingulata pristissini 1
cingulata cingulata 1
bengalensis 2
bengalensis 1
intermedia 5
intermedia 1
Littorinopsis
intermedia 2
intermedia 7
intermedia 4
intermedia 6
intermedia 3
philippiana 1
philippiana 2
pallescens 3
pallescens 2
pallescens 1
angulifera 1
angulifera 3
angulifera 2
angulifera 4
scabra 6
scabra 4
scabra 5
scabra 2
scabra 3
scabra 1
Geographical distribution
Eastern Pacific (EP)
Eastern Atlantic (EA)
Western Atlantic (WA)
Indo-West Pacific (IWP)
EPA
0.1 subst/site

3. Results

3.1. Phylogenetic analysis

The phylogenetic trees constructed from the separate gene datasets are shown in Fig. 1. Resolution of deeper branches of the phylogeny was greatest in the 28S tree, while the 12S analysis resolved more terminal and species-level groupings, and COI yielded the best resolution at all but the deepest levels. There was only one case of conflict between strongly supported (PP > 95%) topologies among the three single-gene trees (the position of *L. vespacea* in 12S and COI trees). There were a few examples of conflicting clades with weaker support (95% > PP > 90%).

In the 28S tree (Fig. 1A) *L. aberrans* was sister to the remaining ingroup taxa (PP = 98%). There was marginal support for the *Littorinopsis* clade (PP = 91%), but the *Protolittoraria* clade was not supported (PP = 62%, with *L. (Lamellilitorina) albicans* included on a long branch). There was marginal support for a single clade that included *Littoraria* and *Palustorina* (PP = 91%), and maximal support for each of these subgenera (although *L. vespacea* was not included in the latter).

There was a basal polytomy in the 12S tree (Fig. 1B). Within this a single clade included *L. vespacea* and all members of *Protolittoraria*, *Littoraria* and *Littorinopsis* (PP = 97%; unsupported in any other single or combined analysis), and the last two of these received high support (PP = 99% and 100%, respectively). *Littoraria vespacea* was included at the base of the *Littoraria* clade, in contrast to the other single-gene and combined trees (in which its position was unresolved, or with *Palustorina*), but with low support (PP = 89%). Surprisingly, neither the *Palustorina* clade, nor this same group without *L. vespacea*, was supported by the 12S analysis.

Of the single-gene analyses, only that of COI gave strong support to a clade of *Protolittoraria* and *Littorinopsis* (PP = 99%). All the four large subgenera were recovered, with high support for *Littorinopsis*, *Protolittoraria* and *Palustorina* (including *L. vespacea*) (PP = 100%, 95%, 99%, respectively), and low support for *Littoraria* (PP = 86%). All species-level taxa received maximal support, except *L. cingulata* (PP = 94%), *L. melanostoma* and *L. coccinea* (both paraphyletic), and *L. ianthostoma* (represented by a single specimen in all analyses).

In the combined tree (Fig. 2) all subgeneric clades received maximal support, and this result was used to reassign two species (see Section 4). The two monotypic subgenera, *L. (Bulimilittorina) aberrans* and *L. (Lamellilitorina) albicans*, were, respectively, the first and second basal branches of the ingroup (PP = 100%). The remaining four subgenera formed two clades, *Protolittoraria* plus *Littorinopsis* with maximal support, and *Palustorina* plus *Littoraria* with marginal support (PP = 91%). Support for nominal species was 100% in all cases, except for the paraphyletic *L. coccinea* and the two species represented by a single specimen (*L. conica*, *L. ianthostoma*). Problems of species status are summarized in Table 2. Within the clades *Littoraria*, *Littorinopsis* and *Protolittoraria* there was strong support for several sister-species relationships, but resolution within *Palustorina* was poor.

3.2. Divergence times and rates of diversification

Divergence times for each species-level taxon (see Section 4) estimated by BEAST are shown in Fig. 3. The topology of the chronogram agreed with that obtained by MrBayes for all clades for which the latter program found more than 95% support, and the posterior probabilities calculated by BEAST were similar, neither consistently higher nor lower (Fig. 2).

3.3. Geographical distributions

The geographical distributions of the *Littoraria* species are listed in Table 1, and superimposed on the combined phylogenetic tree in Fig. 2. The subgenus *Littoraria* is restricted to the Atlantic and Eastern Pacific (EPA) region. *Palustorina* is found only in the Indo-West Pacific (IWP) region. *Protolittoraria* occurs in the IWP, but with one subspecies, *L. pintado pullata*, resident in the Eastern Pacific (EP). *Littorinopsis* is found in the IWP, with the exception of *L. angulifera* in both Eastern and Western Atlantic (EA and WA). These two exceptions are two of the three cases of sister-relationships between taxa in the EPA and IWP; the third is that between the subgenera *Littoraria* and *Palustorina* (but with marginal support, PP = 91% in combined-gene tree, PP = 96% in BEAST analysis).

There are two examples of trans-Isthmian sister-species pairs (i.e. sisters in EP and WA) within the subgenus *Littoraria*: *L. rosewateri* and *L. tessellata* (PP = 98%), and *L. variegata* and *L. irrorata* (PP = 100%). Distribution maps of the five well-supported sister-species and terminal clades within IWP *Littorinopsis* are shown in Fig. 4 (placement of *L. delicatula* as sister to *L. pallescens* as suggested by Reid, 2001, adds a sixth). The only remaining sister-species relationship with strong support anywhere in the combined tree is that between *L. glabrata/coccinea* (in future to be considered a single species, see Section 4) and *L. undulata*, with broadly overlapping distributions spanning the entire width of the Indian and Pacific Oceans (maps not shown). Of the five well-supported pairs of sister-species in the IWP, in only one (*L. luteola* and *L. lutea*, Fig. 4) are the ranges entirely allopatric.

4. Discussion

4.1. Species-level classification

The genus *Littoraria* has been the subject of detailed taxonomic work, and monographic revisions have appeared for the IWP (Reid, 1986a, 2001; Stuckey and Reid, 2002) and EP (Reid, 1999a), together with critical listing of the remaining species (Reid, 1986a, 1999b). Diagnostic characters include not only shells, but also a suite of characters of the reproductive anatomy, of which one, the shape of the penis, has been proposed as a species-recognition character (Reid, 1986a). As a result, the number of species has been precisely estimated at 39, plus two subspecies. Almost all these taxa are represented in our analysis. One exception is *L. delicatula* from the Bay of Bengal, a possible sister taxon of *L. pallescens* (Reid, 2001). The other is *L. flammea* from China, the likely sister-species of *L. melanostoma* (Reid, 1986a), which has not been collected since the early nineteenth century and is believed to be extinct (Carlton, 1993).

Molecular techniques have increased the number of evolutionarily significant units (ESUs; Moritz, 1994) in several taxonomically well-known groups of marine molluscs by between 20% and 59% (Meyer, 2003, 2004; Williams and Reid, 2004; Frey and Vermeij, 2008). Few such studies, however, have attempted to assess the species status of the ESUs (Reid et al., 2006). Within *Littoraria* there is evidence of morphological variation, and of very wide and sometimes disjunct distributions, in several morphospecies and, where possible, we included more than two samples of such species in the expectation of finding evidence for additional ESUs or cryptic species. These cases are listed in Table 2, together with a summary of molecular evidence and preliminary conclusions on species status. Since investigation of species boundaries was not our primary aim, intraspecific sampling was limited and these cases require further study. Meanwhile, we suggest that ESUs that fulfill the following criteria are distinct species (Avise and Wollenberg, 1997; Knowlton, 2000; Avise, 2004; Reid et al., 2006): well-supported

Table 2
Summary of species problems in *Littoraria*, including geographical distributions, morphological differences and molecular evidence. Data on distribution and morphology from Reid (1986a, 1999a, 2001) unless otherwise quoted. Under each gene are listed the posterior probability values (%) for the putative component clades within the nominal species, or for each member of a species complex, or for monophyly of the nominal species itself (*L. melanostoma*); 'no' indicates no support for monophyly; a dash indicates inapplicability when only a single member of a putative clade was sampled. The K2P distance is the maximum between putative clades or among members of single species (%). *Abbreviations: n*, number of samples in nominal species or species complex; K2P, Kimura two-parameter genetic distance for COI sequences.

Species problem	*n*	Distribution	Morphological differences	Molecular evidence				Preliminary conclusions and additional evidence
				28S	12S	COI	K2P	
L. angulifera: E vs W Atlantic	4	Disjunct: Florida to Brazil; W Africa	Not consistent (Merkt and Ellison, 1998)	no	no	no	2.81	Single species; supported by morphometric analysis (Merkt and Ellison, 1998)
L. articulata: W Australia vs E Australia plus Asia	3	Continuous W–E Australia; Asia disjunct	Shell sculpture	no	no	no	3.95	Single species; allozymes but not COI show genetic structure within Australia (Stuckey, 2003)
L. articulata: China vs Australia plus SE Asia	3	Ditto	Not consistent	no	–, 95	–, 100	4.11	Single species with genetic structure; Chinese sample divergent despite lack of obvious disjunction from SE Asia
L. carinifera: Indian O. vs Pacific O.	3	Continuous: India–Philippines, but contact only via Straits of Malacca	Shell sculpture	no	no	–, 87	2.97	Single species with likely genetic structure
L. cingulata cingulata vs *L. c. pristissini*	4	Disjunct: NW Australia; Shark Bay	Shell sculpture	no	no	no	0.61	Single species. Allozymes show isolation by distance (Johnson and Black, 1998)
L. coccinea vs *L. glabrata*	4	Disjunct: Pacific O.; Indian O. (Rosewater, 1970)	Shell color	82, 100	no	no, 99	0.93	Single species (names retained as subspecies). Some evidence of reciprocal monophyly, but distance low
L. filosa: W vs E Australia	2	Continuous: W–E Australia	Shell sculpture and color; possibly penis (Reid, 2001)	–	–	–	3.00	Single species, but allozymes and COI show genetic structure (Stuckey, 2003)
L. intermedia	7	Wide, continuous: IWP	Shell sculpture and color	no	no	92	1.07	Single species; Red Sea sample slightly divergent
L. melanostoma	2	Continuous: China–India	Not consistent	100	no	no	10.48	Large distance and lack of consistent monophyly suggests possible cryptic species
L. pallescens	3	Wide, continuous: IWP	Not consistent	100	100 no, 78	no	3.46	Single species; sample from Sabah divergent
L. pintado pintado vs *L. p. pullata*	6	Disjunct, wide: IWP; E Pacific	Shell color	97, 95		no	0.77	Single species (names retained as subspecies). Some evidence of reciprocal monophyly, but distance low
L. scabra	6	Wide, continuous: IWP	Not consistent	no	no	96	0.61	Single species; no obvious geographical patterns
L. strigata, *L. articulata*, *L. sinensis*	9	Partly sympatric: central IWP	Penis; shells sometimes indistinguishable	100, 100, 54	99, 98, 100	100, 100, 100	11.01	Three species
L. undulata	5	Wide, continuous: IWP (Rosewater, 1970)	Not consistent	no	no	no	0.31	Single species

monophyly in both the nuclear gene (28S) and at least one of the mitochondrial genes; if no differentiation of nuclear gene then at least one consistently diagnostic morphological character (assumed to have a genetic basis); genetic distance (K2P for COI) greater than 4.15%. This genetic distance is that between the closest pair of certain sister-species, *L. filosa* and *L. cingulata*, which fulfill the genetic and morphological criteria and, in addition, are sympatric over much of their range and therefore likely to be not only phylogenetic, but also biological, species (Knowlton, 2000). For comparison, equivalent distances between sister-species of *Echinolittorina* are usually greater than 6%, but in three cases fall between 2.61% and 4.53% (Reid et al., 2006; Reid, 2007, 2009).

Littoraria angulifera shows the widest geographical disjunction, on either side of the Atlantic Ocean, but without consistent morphological differentiation; we found no evidence for cryptic species, in agreement with a morphometric study (Merkt and Ellison, 1998). Of those species showing a geographical pattern of morphological variation, with either fully or almost disjunct distributions, we found no strong evidence for additional species (*L. articulata*, *L. carinifera*, *L. cingulata*, *L. filosa*). Several species have very wide distributions, spanning most of the IWP region. Of these, neither *L. scabra* nor *L. undulata* have obvious regional variation in shell morphology, whereas *L. intermedia* and *L. pallescens* exhibit geographical forms (Reid, 1986a), but in none of these was there a distinct genetic pattern that suggested species-level subdivision (Table 2). *Littoraria pintado* shows the widest distribution of all, across the full width of the Indian and Pacific basins, but with disjunct occurrences in the western Indian Ocean, the northwestern Pacific, and the eastern Pacific Ocean (Reid, 1999a). While no differentiation can be seen within the IWP, the eastern Pacific form has been recognized as a subspecies, *L. pintado pullata*, by virtue of its distinctive shell color pattern. Reciprocal monophyly of the western and eastern forms was found for 28S sequences alone (weak evidence for 12S; Table 2) and the genetic distance for COI was only 0.77%, suggesting that this is a single species within which weak differentiation corresponds to the subspecies recognized on morphological grounds. The pair *L. coccinea* and *L. glabrata* occupy the western Pacific and western Indian Oceans, respectively, and have traditionally been regarded as distinct, although they can be separated only by shell color (Rosewater, 1970; Reid, 1986a). As in *L. pintado*, there is some evidence for reciprocal monophyly in 28S and a mitochondrial gene (Table 2), but the genetic distance is similarly low (0.93%). For consistency, we recommend that this pair should likewise be considered a single species (*L. coccinea*), within which subspecific names can be used for the recognizable, geographically separated isolates. Only in the single case of *L. melanostoma* was there a suggestion of cryptic species; only two specimens were sequenced, but showed a genetic distance for COI of 10.48% and monophyly was supported only by the nuclear gene. The three morphologically similar species *L. articulata*, *L. sinensis* and *L. strigata* can be differentiated only by penial shape (Reid, 2001), but were confirmed as distinct species by genetic distance and by monophyly in each gene tree.

In summary, the species defined by our molecular study conform closely to those recognized by traditional morphology, except that *L. coccinea* and *L. glabrata* are not distinct species, and with a suggestion of a possible cryptic species in *L. melanostoma*.

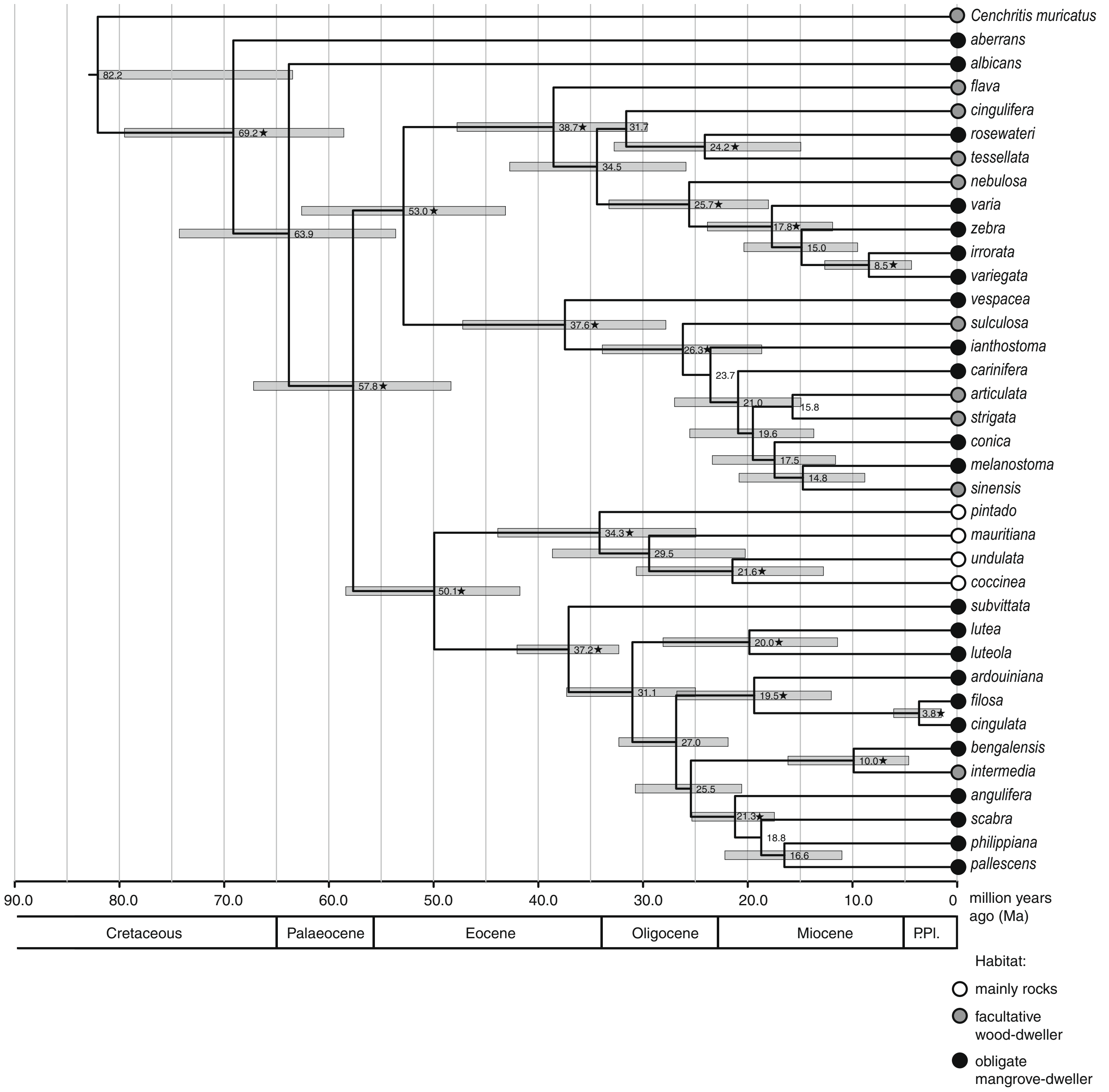


Fig. 3. Chronogram generated by BEAST for species-level taxa of *Littoraria*, calibrated using four fossil records (see Material and Methods). Asterisks following ages of nodes indicate clades with posterior probabilities greater than 95% (see also Fig. 2). Error bars for node ages are 95% highest posterior density intervals; only half of the error bar of the basal node is shown. The topology is shown as fully resolved, but those clades with less than 50% support have no error bars. Habitats of the species (modified from Reid, 1999b) are superimposed.

4.2. Phylogeny and subgeneric classification

In a large genus of worldwide distribution, subgeneric division serves a useful heuristic purpose. The current scheme is the result of three parsimony analyses of morphological characters (Reid, 1986a, 1989, 1999b). The latest of these recognized three major clades defined by clear synapomorphies: *Littorinopsis* (synapomorphy: ovoviviparous brooding in mantle cavity with planktotrophic development); *Palustorina* (synapomorphy: flagellum-like pseudotrich of paraspermatozoa); and the basal *Protolittoraria* (synapomorphies: smooth shell and egg capsule shape). In addition there were two monotypic subgenera of uncertain affinity (*Lamellilitorina* with autapomorphy of large planktotrophic protoconch; *Bulimilittorina* with autapomorphies of ovoviviparity with intracapsular metamorphosis, and unique penial glands), while the remaining species were contained in the subgenus *Littoraria*, a paraphyletic assemblage. Inclusion of the newly described species *L. ianthostoma* in the analysis yielded an uncertain placement in *Littoraria* (Stuckey and Reid, 2002). A phylogenetic analysis of allozyme frequencies of 11 mainly Australian species gave some support to the three main subgenera and to the basal placement of *Protolittoraria* (Inness-Campbell et al., 2003). In contrast, *Protolittoraria* appeared in a terminal position when four *Littoraria* species were included in a subgeneric-level molecular analysis of the subfamily

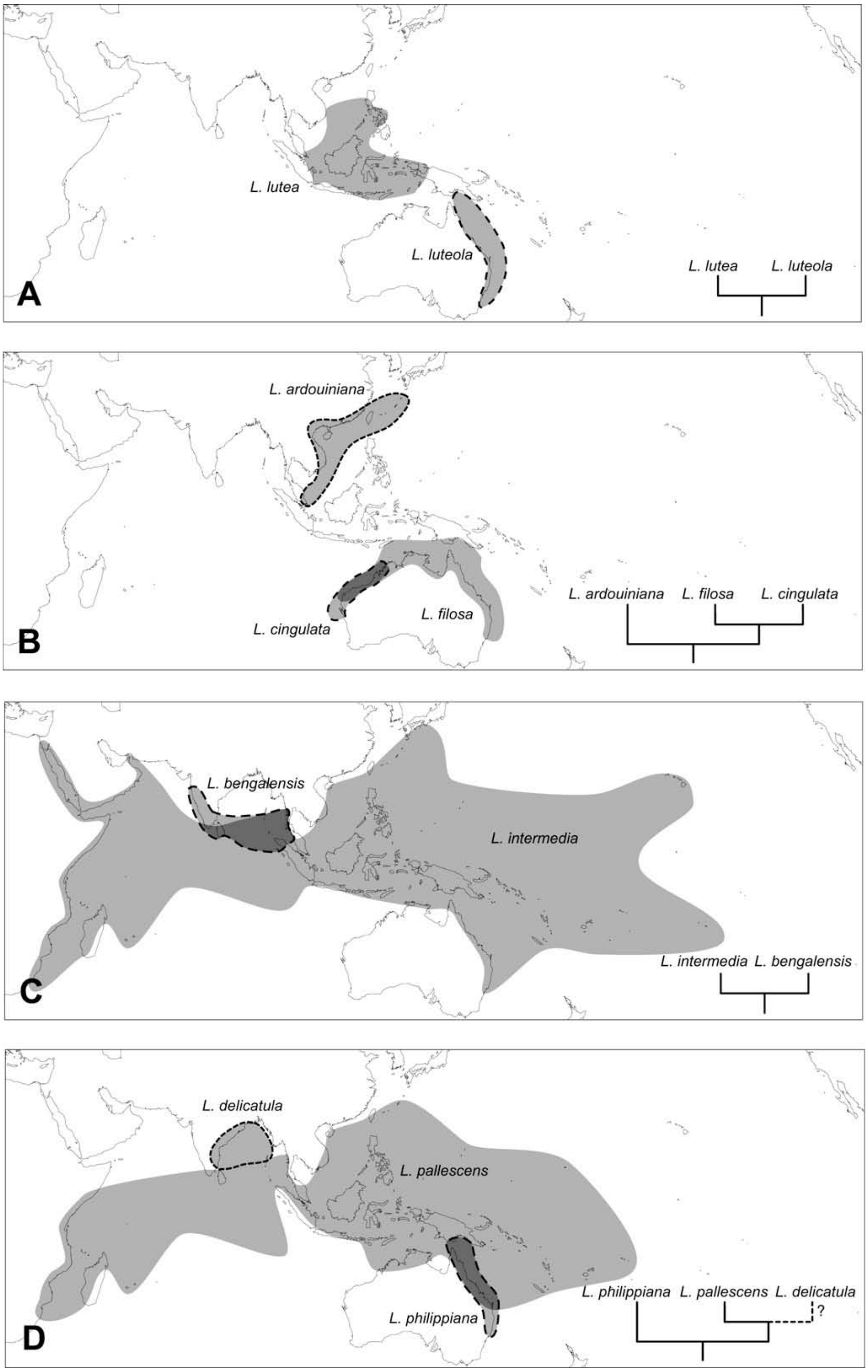


Fig. 4. Geographical distributions for selected sister-species pairs and terminal clades of *Littoraria* in the Indo-West Pacific region. Inset trees indicate topology only (see Fig. 2 for branch lengths and support values). *Littoraria delicatula* was not analysed in this study; its possible sister-relationship with *L. pallescens* was suggested by Reid (2001). Where distributions overlap, dotted and dashed outlines are used for clarity. Distributions from Reid (1986a, 2001).

Littorininae, based on partial sequences of two nuclear and two mitochondrial genes (Williams et al., 2003).

The present study has largely resolved the composition and relationships of the morphologically-based subgeneric divisions (Fig. 2). The existing six subgenera are sufficient to describe the principal clades, and only two species need to be reassigned. The 'basal' taxon (i.e. sister to all the rest) is *L. (Bulimilittorina) aberrans*, and the next *L. (Lamellilitorina) albicans*, both morphologically aberrant species of hitherto unknown affinity. *Protolittoraria* is confirmed as the sister taxon of *Littorinopsis*. The subgenus *Littoraria* is shown to be monophyletic for the first time. Of the existing subgenera, *Palustorina* has been considered the most robustly supported, on the basis of a unique and unequivocal synapomorphy, the pseudotrich of the paraspermatozoa (Reid, 1986a, 1989, 1999b; Healy and Jamieson, 1993; Stuckey and Reid, 2002). It is therefore unexpected that this clade was not recovered in any of the single- or combined-gene analyses. Instead, a larger group (formed by the addition of *L. vespacea* and *L. ianthostoma*) appeared with strong support in the combined analysis, and we recommend that *Palustorina* should now be used in this sense, despite the absence of any known morphological synapomorphy. Interestingly, this group shares a remarkable similarity in penial shape, contrasting with the diversity within other subgenera, possibly implying that this character is less important in the specific mate-recognition system of *Palustorina*.

4.3. Biogeography: origins and global patterns of vicariance and dispersal

For groups with early Cenozoic or older origins and a poor fossil record, the area of origin is unlikely to be discoverable. Attempts based on reconstruction of ancestral areas on molecular phylogenies have often supported classical ideas of origination in the tropical Tethyan Realm (Kirkendale and Meyer, 2004; Latiolais et al., 2006; Frey and Vermeij, 2008; Reid et al., 2008). However, others have shown paraphyletic basal lineages in the Americas (or EPA region) (Anderson, 2000; Williams and Reid, 2004; Williams, 2007; Williams and Duda, 2008), which is consistent with fossil evidence that demonstrates origination of some gastropod families in tropical America during the Eocene (Vermeij, 2001). For *Littoraria* it is marginally more parsimonious (4 vs 5 steps; Fig. 2) to reconstruct the EPA rather than IWP as the ancestral area (because both the outgroup and the basal *L. aberrans* are from EPA). Nevertheless, this argument is weak, for the pattern is likely to have been modified by extinction and there is no supporting fossil evidence. At least, the existence of two narrowly-distributed relictual species on opposite sides of the globe (*L. aberrans* in Panama; *L. albicans* in Borneo) hints at an ancient (Late Cretaceous or Palaeocene, Fig. 3) global distribution.

The present isolation of the four great biogeographical regions of the marine tropics is the result of progressive vicariance of the circumtropical Tethyan Realm throughout the Cenozoic, and from this tectonic history testable predictions can be made of relationships among regions and of ages of divergence (e.g. Meyer, 2003; Floeter et al., 2008). In *Littoraria* there are three cases of sister-relationship between clades in the IWP and EPA (Fig. 2). One is between the amphi-Atlantic *L. angulifera* and up to three IWP species (*L. scabra*, *L. pallescens*, *L. philippiana*). Since these four are all morphologically similar, the presence of *L. angulifera* in the Atlantic has been ascribed to a recent (Pliocene) migration from the IWP around the tip of southern Africa (Vermeij and Rosenberg, 1993). However, our estimate of the age of *L. angulifera* is 21.3 ± 3.9 Ma, close to that of the final closure of the Tethyan Seaway at 18–19 Ma (Vrielynck et al., 1997; Rögl, 1998; Harzhauser et al., 2002, 2007), as predicted if divergence was the result of Tethyan vicariance. The fossil record supports this interpretation, because there are specimens resembling *L. angulifera* in the Early Miocene of France (*L. grateloupi*; Reid, 1999b) and of Panama (see Section 2). In contrast, most molecular estimates of the ages of putative Tethyan divergences are much older than the closure of the seaway, in the range 21–46 Ma (Bellwood et al., 2004; Williams and Reid, 2004; Duda and Kohn, 2005; Williams, 2007; Frey and Vermeij, 2008; Williams and Duda, 2008; Malaquias and Reid, 2009). This is also the case for the second IWP/EPA relationship within *Littoraria*, that between the subgenera *Palustorina* and *Littoraria*, estimated at 53.0 ± 9.8 Ma. Besides the errors associated with the estimation of the tree, evolutionary rates and the vagaries of the fossil record (discussed by Wilke et al., 2009), a likely explanation for this discrepancy is the extinction of true sister lineages in the EPA (Paulay, 1997; Williams and Reid, 2004; Williams, 2007). Alternatively, the final closure of the Tethyan Seaway may not have been the cause of vicariance. An earlier vicariant event was the conjunction of the northward-moving Indian plate with Asia during the Eocene (Ali and Aitchison, 2008), which divided the Tethyan Realm into distinct eastern and western biogeographic regions (Harzhauser et al., 2002, 2007). Another was the climatic cooling that occurred at the end of the Eocene, potentially resulting in extinction of tropical species (including mangroves, as discussed below) in the high-latitude Tethyan Seaway (Plaziat et al., 2001; Bowen, 2007; Zanazzi et al., 2007).

The third case of sister-relationship between the IWP and EPA occurs between clades within *L. pintado*, traditionally designated as subspecies. *Littoraria pintado pintado* has a disjunct distribution in the western Indian Ocean and northwestern Pacific, while *L. p. pullata* occurs at Cabo San Lucas (Mexico) and on oceanic islands (excluding the Galapagos) in the eastern Pacific (Reid, 1999a). The close genetic distance (Table 2) indicates a very recent divergence, likely during the Pleistocene, which is consistent with the age of the islands on which *L. p. pullata* occurs (Reid, 1999a). Since this is later than the uplift of the Isthmus of Panama, colonization of the eastern Pacific region can only have been by eastward dispersal from the IWP, across the formidable 5000-km expanse of open ocean that constitutes the Eastern Pacific Barrier (Vermeij, 1987; Lessios and Robertson, 2006). This refutes the alternative hypothesis of an ancient Tethyan origin (Reid, 1986a). No other littorinid species maintains viable populations on either side of this oceanic barrier. However, two other *Littoraria* species, *L. undulata* and *L. coccinea*, occasionally cross in the same direction to reach Cocos Island and Clipperton Atoll (Reid and Kaiser, 2001). Together, these are three of the four members of subgenus *Protolittoraria*, which suggests an enhanced dispersal capacity in this clade. The larval shells of *Protolittoraria* species are no larger than those of other *Littoraria* species, so this capacity may rest in extended larval competence rather than prolonged growth (see Reid and Kaiser, 2001, for further discussion).

The uplift of the Isthmus of Panama is the most well studied case of tectonic vicariance in the marine realm, and took place over at least 15 Myr before completion at 3.4 Ma (Coates and Obando, 1996; Kirby et al., 2008; Lessios, 2008). Molecular estimates of divergence times across this barrier span a wide range, from 3 to over 30 Ma (Knowlton and Weigt, 1998; Marko, 2002; Bellwood et al., 2004; Williams and Reid, 2004; Duda and Kohn, 2005; Frey and Vermeij, 2008; Lessios, 2008; Malaquias and Reid, 2009). Again, extinction and more ancient vicariant processes may be responsible for this discordance. Of three trans-isthmian relationships in *Littoraria*, two fall near the older limit of this range (*L. rosewateri* and *L. tessellata* at 24.2 ± 9.0 Ma; *L. nebulosa* and a clade of four species at 25.7 ± 7.7 Ma). The third, between *L. variegata* in the EP and *L. irrorata* in the southeastern United States, is more interesting, because it is one of the two youngest interspecific divergences in the genus. It is expected that species occupying shallow-water sediments and mangrove habitats should be among the last to have been separated during the gradual shoaling of the isthmus (Knowlton and Weigt, 1998; Hurt et al., 2008). Fossils of *L. irrorata* have been recorded from the Late Miocene and Pliocene (see Section 2) and were used for calibration in the BEAST analysis. The resulting estimate of divergence was 8.5 ± 4.1 Ma, so even this divergence does not appear to represent a case of latest possible separation of a true geminate pair. The major extinctions that occurred following the uplift of the isthmus, predominantly in the WA and peaking at 1–2 Ma (Vermeij and Rosenberg, 1993; Jackson et al., 1993; Allmon et al., 1996; O'Dea et al., 2007) could be responsible for the rarity of such geminate pairs (Marko, 2002). *Littoraria irrorata* is the most temperate in distribution within the genus, and can be added to the examples of northward migration from the tropical Gatunian to temperate Caloosahatchian province during the Pliocene (Vermeij, 2005).

Within the Atlantic, the principal biogeographic barriers for shallow-water marine taxa are the width of the ocean itself and the Amazon Barrier (Floeter et al., 2008; Malaquias and Reid, 2009). Neither has had a major influence on the history of *Littoraria*; *L. angulifera* occurs on both sides of the Atlantic, and both this species and *L. flava* are distributed across the Amazon Barrier. Only *L. cingulifera* is restricted to the eastern Atlantic, and both its phylogenetic and biogeographic relationships are equivocal (Fig. 2). This contrasts with *Echinolittorina*, in which diversification has been influenced by both these barriers (Williams and Reid, 2004; Reid, 2009), and points to greater dispersal and larval tolerance in *Littoraria*.

Within each of the four major tropical marine regions, *Littoraria* species display a high degree of range overlap, so that little geographical signal remains to suggest causes of diversification, as discussed below. In the largest region, the IWP, six of the 25 species show very wide distributions from Africa to the central Pacific or further east, therefore exceeding the range of any of the 26 *Echinolittorina* species in the region (Reid, 2007). This again suggests wider potential for dispersal in *Littoraria*; although duration of larval development is only one of the determinants of range size, the two are often correlated (Kohn and Perron, 1994; Meyer, 2003). There are several competing models to explain the causes of diversification and resulting patterns of diversity within the IWP (e.g. Bellwood and Wainwright, 2002; Williams and Reid, 2004; Bellwood and Meyer, 2009). Regarding its timing, there is growing evidence from both fossils (Wilson and Rosen, 1998; Renema et al., 2008) and molecular phylogenies (Williams, 2007; Williams and Duda, 2008; Frey and Vermeij, 2008) for an increased rate of diversification in the central IWP between the Late Oligocene and Early Miocene (16–25 Ma), corresponding to the tectonic impact of the Australian plate with Southeast Asia. While many estimates of divergence times among *Littoraria* from the IWP fall within this interval (Fig. 3), the rate of diversification at this time is not significantly greater in the IWP clades (S.T. Williams, unpublished).

4.4. Radiation and speciation in the mangrove environment

There has been debate about whether the characteristic molluscs of the mangrove environment are strictly associated with this habitat, or simply avail themselves of any hard substrate in sheltered inlets and estuaries, with otherwise soft sediments (Plaziat, 1984; Plaziat et al., 2001). Some members of the community (e.g. some Muricidae and *Nerita*) are more frequent on rocky shores and fall into the latter category. However, the Potamididae show a range of adaptations for life in mangroves (including shell construction, diet and behavior) and it has been argued that for most of them the association is obligate (Reid et al., 2008). Reconstruction of ancestral habitats on a phylogeny of potamidids suggests that the common ancestor was a ground-dwelling inhabitant of mangroves, and that tree-climbing and independence from mangroves are both derived conditions (Reid et al., 2008). *Littoraria* species likewise show a close association with mangroves although, unlike potamidids, they cannot exist on soft substrates. The great majority are found only on the trunks, branches and foliage of mangrove trees and other salt-tolerant shrubs and grasses; these can be described as ‘obligate mangrove-dwellers’. Some have a broader habitat range, including mangroves, driftwood and sheltered rocks; these ‘facultative wood-dwellers’ are members of subgenera *Littoraria* (*flava*, *cingulifera*, *tessellata*, *nebulosa*), *Palustorina* (*sulculosa*, *articulata*, *strigata*, *sinensis*), *Littorinopsis* (*intermedia*) and *Protolittoraria* (Reid, 1999b). The last of these is remarkable because all of its four members occur mainly on rocky shores, sometimes even of moderate exposure, two on rocks alone (*L. pintado*, *L. mauritiana*) and two sometimes also on driftwood (*L. undulata*, *L. coccinea*), but extremely rarely on mangroves. An earlier morphological phylogenetic analysis retrieved *Protolittoraria* as the basal branch of *Littoraria*, and it was argued by outgroup comparison that the ancestral habitat for the genus was therefore on rocky shores (Reid, 1999b). The present topology discounts this (as hinted by Williams et al., 2003), revealing *Protolittoraria* as a derived clade in which obligate mangrove-dwelling has been lost (Fig. 3). The distinction between obligate and facultative mangrove-dwellers is not sharp, and even varies across the range of some widespread species (e.g. *L. intermedia*; Reid, 1986a, 2001). The most that can be said about the ancestral habitat of *Littoraria* is that it included wood and/or mangroves. Facultative wood-dwelling is shared by its sister group *Cenchritis*, and therefore probably arose in the common ancestor of both. This ability appears to be associated with an additional cutting edge of the radula (the ‘rachidian hood’) that is phenotypically induced by grazing on wood substrates in both genera (Reid and Mak, 1999). This line of phylogenetic evidence cannot determine whether the earliest radiation of *Littoraria* might have been an adaptive one, connected with an obligate mangrove-dwelling habit.

Further evidence can be sought in the fossil record. However, identification of ancient mangrove communities on the basis of fossils is seldom unequivocal (Plaziat, 1995). Only one fossil ‘littorinid’ has been suggested as a diagnostic member of a mangrove fauna, *Littorinopsis miodelicatula* Oyama, 1950 from the Miocene of Japan, which is found together with a typical mangrove association including *Telescopium*, *Rhizophorimurex* and *Geloina* (Oyama, 1950). Nevertheless, this is not a *Littoraria*; details of the aperture and sculpture reveal that it is a member of Trochidae (D.G. Reid, unpublished; based on material in BMNH and figures in Oyama, 1950; although ‘*Littorinopsis miodelicatula*’ figured by Ozawa et al., 1986, from another Japanese mangrove fauna of the same age is a likely *Littoraria*). There is no direct evidence for mangrove association in the earliest, Eocene, fossils of *Littoraria* (Reid, 1999b). Their location in the Tethyan Realm, and the estimates of divergence times of the basal branches of the *Littoraria* phylogeny (spanning Late Cretaceous to Eocene; Fig. 3), are, however, consistent with the Tethyan origin of the principal mangrove plant genera during the Palaeocene (Ellison et al., 1999; Plaziat et al., 2001). One other possible parallel between the historical biogeography of *Littoraria* and mangrove plants can be suggested. Plaziat et al. (2001) have documented the disappearance of Rhizophoraceae from Europe during the cooling phase of the Middle Eocene and the eradication of almost all mangroves from the European Tethyan Realm during the climatic crisis at the close of the Eocene. They suggested that this climatic vicariance, rather than the later tectonic closure of the Tethyan Seaway, was the origin of the disjunction between the mangrove floras of the EPA and IWP. If the same were true of *Littoraria*, this could explain the 43.2–62.8 Ma estimate of the divergence of *Littoraria* and *Palustorina* (in EPA and IWP, respectively), a range that overlaps the middle of the Eocene. If fossils are correctly identified, the subgenus *Littorinopsis* was also present in Europe as early as the Middle Eocene (see Section 2), but left no direct descendents in the modern EPA fauna. During the Early and Middle Miocene warm interval, *Avicennia* reappeared in Europe (Plaziat, 1995; Plaziat et al., 2001), and this may have permitted the migration of the ancestor of *L. angulifera* from the IWP to the EPA before the closure of the Tethyan Seaway (estimate 17.4–25.2 Ma). These speculative correlations should be tested as further details emerge of the fossil records of mangroves and *Littoraria*.

One of our aims was to test the ways in which the mangrove habitat could have influenced the speciation processes of its fauna. Arguments based on sensitivity of mangroves to glacio-eustatic sea-level change (Woodroffe and Grindrod, 1991; Sun et al., 2000) and on late separation of mangrove habitats on either side of the Panama Isthmus (Knowlton and Weigt, 1998; Hurt et al., 2008) suggest that speciation of mangrove fauna might have taken place relatively recently, during the Pleistocene. Our results suggest that this is not generally the case; only one estimate of sister-species divergence overlaps the Pleistocene (*L. filosa* and *L. cingulata*, 1.6–6.2 Ma; Fig. 3). Recent *Littoraria* species are predominantly of Pliocene to Miocene age, as reported in many groups of molluscs and fish (Bellwood and Meyer, 2009), from habitats including reefs (Barber and Bellwood, 2005; Read et al., 2006), rocky shores (Williams and Reid, 2004; Frey and Vermeij, 2008) and shallow lagoons (Malaquias and Reid, 2009).

Useful comparison can be made with the littorinid genus *Echinolittorina*, which is of comparable diversity (60 species worldwide), tropical distribution and life history, but is restricted to rocky shores. As in *Echinolittorina* the mode of speciation in *Littoraria* appears to have been allopatric, with six of the seven known sister-species pairs retaining some geographical signal (Fig. 4). Nevertheless, most such pairs show partial geographical overlap (exceptions are the two trans-isthmian pairs in Fig. 2 and the IWP pair in Fig. 4A), and geographical signal largely disappears at deeper levels within the phylogeny. Thus there is complete overlap among the four members of the subgenus *Littoraria* in the EP (Reid, 1999a) and up to six species of *Palustorina* overlap broadly in the central IWP (Reid, 1986a, 2001). In contrast, among *Echinolittorina* species strict allopatry is usually maintained between sister pairs, and often through up to three branching points of the phylogeny (Williams and Reid, 2004). It seems unlikely that this indicates any difference in speciation mode; there is, for example, no specialization of snails on particular tree species (Reid, 1985), which could have driven small-scale ecological or sympatric speciation. Instead, allopatry is likely to be more transient in *Littoraria*. This may partly be a consequence of the occurrence of most species on continental margins (as noted in *Tegula* by Hellberg, 1998); along the continuous coastline of the EP even *Echinolittorina* species show moderate overlap (Williams and Reid, 2004). In the central IWP the distributions of marine species along the broad continental shelves must have been profoundly disrupted when these were exposed during Plio-Pleistocene sea-level fluctuations (Voris, 2000), so the extensive geographical overlap among the nine members of *Palustorina* is not unexpected. Furthermore, the scale of dispersal in the genus *Littoraria* may be greater for, as argued earlier, larval life probably exceeds the 3–4 weeks recorded for *Echinolittorina*, and in addition mangrove plant debris may offer the potential for dispersal of adults by rafting. There are no narrow-range endemics on distant oceanic islands as are found in *Echinolittorina*. This is not only due to the absence of mangroves across large areas of the archipelagic Pacific basin (Spalding et al., 1997), for even the rock-dwelling members of *Protolittoraria* have not diverged on remote islands, only showing minor (intraspecific) divergence at the very largest oceanic scales (between Indian and Pacific Oceans in *L. coccinea*/*glabrata*, and between IWP and EP in *L. pintado*). The low diversity (4 species) of *Protolittoraria*, wide geographical extent, and genetic structure on the largest scale are all typical features of marine organisms with a long larval phase and high gene flow (Lessios et al., 2001; Paulay and Meyer, 2002; Meyer, 2003). It has been suggested that interspecific competition on rocky shores may limit sympatric occurrence among recently-diverged *Echinolittorina* species (Williams and Reid, 2004; Reid, 2007), but in mangrove environments *Littoraria* species occur at low densities under the influence of high levels of predation (Reid, 1985, 1992), and up to 10 species occur sympatrically in the central IWP (Reid, 1986a).

It is increasingly recognized that occurrence in either oceanic or continental habitats can be an important dimension of the ecological niche of marine species, circumscribing distributions at local and geographical scales (Williams and Reid, 2004; Reid et al., 2006; Lee and Ó Foighil, 2005) and playing a role in parapatric ecological speciation (Rocha et al., 2005, 2008). *Echinolittorina* species provide some of the clearest examples of contrasting oceanic and continental distributions, although because sister-species do not usually differ it is unclear that this has played a role in speciation processes (Williams and Reid, 2004; Reid et al., 2006). Mangroves are associated with eutrophic conditions on continental coasts, but can also be found in more oligotrophic situations on offshore islands (Woodroffe and Grindrod, 1991). The majority of *Littoraria* species lie towards the continental end of the habitat spectrum, although all members of *Protolittoraria* are oceanic. In the subgenus *Littorinopsis* the four most oceanic species are *L. intermedia*, *L. pallescens*, *L. lutea* and *L. scabra* (Reid, 1986a, 2001). The first three of these are each sister to a more continental species with a close or partly overlapping range (Fig. 4), which is the predicted outcome of ecological speciation. Speciation is not always accompanied by ecological differentiation. The three species shown in Fig. 4B are all inhabitants of similarly continental habitats and the sister-relationship between *L. ardouiniana* in eastern Asia and *L. filosa* plus *L. cingulata* in Australia is an example of a repeated biogeographic pattern, in which Asian and Australian clades are separated by the intervening oceanic region of eastern Indonesia (Reid et al., 2006, 2008; Ozawa et al., 2009).

4.5. Evolution of reproductive mode: ovoviviparity in mangroves

The type of larval development is either known, or can confidently be predicted, from oviduct structure and protoconch, in virtually all *Littoraria* species (Reid, 1986a, 1999a, 2001; Stuckey and Reid, 2002; unknown only in *L. flammea*). Members of the subgenera *Lamellilitorina*, *Littoraria*, *Protolittoraria* and *Palustorina* release pelagic egg capsules from which hatch planktotrophic veliger larvae. The duration of larval life has not been recorded directly, but has been estimated at 3–10 weeks, based on comparison of protoconchs with those of other littorinids (Reid, 1986a) and the delay between spawning and recruitment in *L. angulifera* (Gallagher and Reid, 1979). As argued above from distributional data (e.g. the amphi-Atlantic distribution of *L. angulifera*; the amphi-Pacific occurrence of three *Protolittoraria* species), the correct value is probably towards the upper limit of this range, and may be greatest of all in *Protolittoraria*. The frequency, uniformity and phylogenetic distribution of oviparous planktotrophic development among littorinids leave little doubt that this is the plesiomorphic condition in the subfamily Littorininae, although there are hints that the loss of planktotrophy may not be as irreversible as was once thought (Reid, 1989; Williams et al., 2003; Collin et al., 2007).

Members of the subgenus *Littorinopsis* show a derived condition, in which egg capsules have been lost and embryos are retained in the mantle cavity before their release as planktotrophic veligers. Its interpretation as a synapomorphy of the subgenus (Reid, 1986a, 1989) is confirmed by the new molecular phylogeny. This short-term ovoviviparous brooding habit can have little effect on length of larval life (or consequent dispersal ability), because brooding lasts only for a few days. It has been argued that this habit (unique among littorinids) is an adaptation to supralittoral life on the vertical substrate of mangrove trees where predation by crabs is severe; at spawning, females migrate down to the water level during high tide and rapidly release their larvae, reducing the time that would be required for the slow spawning of individual eggs from the oviduct (Gallagher and Reid, 1974), and thus minimizing exposure to aquatic predators (Reid, 1989). The ancestral mangrove- (or wood-) dwelling habit is consistent with this adaptive scenario, and all but one (*L. intermedia*) of the *Littorinopsis* species are classed as obligate mangrove-dwellers.

Only one *Littoraria* species shows non-planktotrophic development—*L. aberrans*. Here the embryos are retained in the mantle cavity throughout their development and released as crawling juveniles (i.e. ovoviviparity with intracapsular metamorphosis; Reid, 1999a). Such a condition could easily be derived from the ovoviviparous planktotrophic state of *Littorinopsis*, although no phylogenetic reconstruction has suggested that *L. aberrans* is a member of that group, and its affinities have remained unknown until now. Its position at the very base of the *Littoraria* clade is, however, unexpected. This demonstrates that ovoviviparity has originated twice in the genus (and, it could be argued, as an adaptation to the mangrove habitat in each case). Nevertheless, because non-planktotrophic species are usually evolutionarily short-lived as a consequence of low dispersal (Jablonski, 1986), it seems unlikely that this trait is as ancient as the lineage leading to *L. aberrans*. The species is rare and of narrow distribution on the Pacific coast of Central America (Reid, 1999a). Instead, extinction may have removed the planktotrophic members of this monotypic subgenus, and they should be sought in the fossil record.

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