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Major adaptive radiation in neritopsine gastropods estimated from 28S rRNA sequences and fossil records

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A well-supported phylogeny of the Neritopsina, a gastropod superorder archaic in origin, radiated ecologically and diverse in morphology, is reconstructed based on partial 28S rRNA sequences. The result (Neritopsidae (Hydrocenidae (Helicinidae + Neritiliidae) (Neritidae + Phenacolepadidae))) is highly congruent with the fossil records and the character distribution of reproductive tracts in extant taxa. We suggest that the Neritopsina originated in subtidal shallow waters, invaded the land and became fully terrestrial at least three times in different clades, by the extinct Dawsonellidae in the Late Palaeozoic and by the Helicinidae and Hydrocenidae in the Mesozoic. Invasion of fresh- and brackish waters is prevalent among the Neritopsina as the Jurassic and freshwater ancestory is most probable for helicinids. The Phenacolepadidae, a group exclusively inhabiting dysoxic environments, colonized deep-sea hydrothermal vents and seeps in the Late Cretaceous or Early Cenozoic. Submarine caves have served as refuges for the archaic Neritopsidae since the Early to Middle Cenozoic, and the marine neritopsine slug *Titiscania* represents a highly specialized but relatively recent offshoot of this family. The Neritiliidae is another clade to be found utilizing submarine caves as shelter by the Oligocene; once adapted to the completely dark environment, but some neritiliids have immigrated to surface freshwater habitats.

Keywords: fossil; molecular clock; Neritopsina; 28S rRNA; phylogeny; adaptive radiation

1. INTRODUCTION

Adaptive radiation—the rise of a diversity of ecological roles and attendant adaptations in different species within a lineage—is one of the most important processes bridging ecology and evolution (Givnish 2000). One of the most significant events in the history of life was the invasion of the land from the sea by higher plants and animals with or without freshwater intermediates (Shear 1991; Vermeij & Dudley 2000). Some animals have invaded, and adapted to, such unique environments as deep-sea hydrothermal vents (Van Dover 2000) and coastal marine caves (Iliffe *et al.* 1984; Iliffe 2000). The history of invasions by individual clades is, however, not necessarily well understood and is often supported by equivocal phylogenetic evidence.

Neritopsina (Neritimorpha), a gastropod superorder, has undergone a major adaptive radiation. The group comprises over 450 living species classified into six families (Fretter 1965; Ponder 1998), and its fossil record extends back at least to the Middle Devonian of *ca*. 375 million years (Myr) ago (Knight *et al.* 1960; Batten 1984) and possibly as early as the Ordovician *ca*. 500 Myr ago (Bandel & Frýda 1999). In tropical and subtropical seas, neritopsines flourish in the intertidal and shallow subtidal zones. They also inhabit totally dark submarine caves (Kase & Hayami 1992; Kano & Kase 2000*b*, 2002) as well as deep-sea hydrothermal vents and oil and gas seeps (Okutani *et al.* 1989; Warén & Bouchet 1993, 2001). Multiple freshwater and terrestrial (including arboreal) invasions have also been suggested (Haszprunar 1988; Holthuis 1995), and neritopsine dispersal has even extended to underground water systems (Sasaki & Ishikawa 2002). Mature neritopsine shells range from minute (less than 1.5 mm) to large (more than 15 cm) and exhibit a variety of shapes and ornamentation including tightly coiled conispiral, patelliform, smooth or spined (Knight *et al.* 1960; Ponder & Lindberg 1997). In one genus, *Titiscania*, the post-larval shell is absent and the animal has undergone complete limacization (Bergh 1890). Given the remarkable morphological and ecological diversity now known to be shown by neritopsines, there emerges an exciting new opportunity to develop and evaluate new concepts and models of adaptive radiation.

The classification of the neritopsine subgroups is, at present, far from being a truly phylogenetic one, in spite of the recent progress in higher-taxa gastropod phylogeny based mainly on soft-part anatomy (Haszprunar 1988; Ponder & Lindberg 1997). No comprehensive hypothesis of subgroup relationships within the Neritopsina has been presented, except that based solely on the fossil record by Bandel (2000). While Holthuis (1995) provided a phylogeny of aquatic neritopsines, convergence in anatomical characters common among terrestrial groups presents a major difficulty in the phylogenetic analysis of this superorder. For instance, the lung of the wholly terrestrial Hydrocenidae and Helicinidae is considered to have evolved independently (Bourne 1911). Any rigorous, non-circular study of adaptive radiation must be based on a phylogeny that has been derived independently of the traits involved in that radiation (Givnish 2000). This places certain limits

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Table 1. Species selected for this study, collection site of specimens and DDBJ accession number.

family	species	locality; habitat	DDBJ no.
(ingroup taxa)			
Neritopsidae	*Neritopsis radula*	Okinawa, Japan; submarine cave, 20 m depth	AB087186
	Titiscania limacina[a]	Okinawa, Japan; intertidal, under deeply embedded rubble	AB087187
Hydrocenidae	*Georissa shikokuensis*	Toyohashi, Japan; terrestrial, on limestone wall	AB087188
Helicinidae	*Pleuropoma* (*Aphanoconia*) sp.	Harpai, Tonga; terrestrial	AB087189
Neritiliidae	*Neritilia rubida*	Arue, Tahiti; freshwater stream, on rubble	AB087190
	Pisulina adamsiana	Sipadan, Malaysia; submarine cave, 9–17 m depth	AB087191
Phenacolepadidae	*Cinnalepeta pulchella*	Kyushu, Japan; intertidal, under deeply embedded rubble	AB087192
Neritidae	*Nerita polita*	Okinawa, Japan; intertidal, rocky shore	AB087193
(outgroup taxa)			
Cocculinidae	*Coccopigya punctoradiata*	Tokyo Bay, Japan; on sunken wood, *ca*. 200 m depth	AB087194
Turbinidae	*Turbo chrysostoma*	Okinawa, Japan; subtidal, rocky shore	AB087195
Viviparidae	*Sinotaia quadratus historica*	Yokohama, Japan; freshwater, ditch	AB087196

[a] Titiscaniidae is synonimized in this study.

on the use of a number of traditional morphological characters (e.g. shell shape, opercular shape). Potentially, molecular studies can provide valuable new evidence concerning relationships within the Neritopsina, as ultrastructural (e.g. spermatological) and karyological studies already have. Several molecular studies of gastropod phylogeny have included neritopsines as operational taxonomic units (OTUs), but only one or two families represent the superorder in these analyses (e.g. Harasewych *et al.* 1997; Rosenberg *et al.* 1997; Winnepenninckx *et al.* 1998; McArthur & Koop 1999; Colgan *et al.* 2000). Also, only a few neritopsine families (Neritidae, Helicinidae and Phenacolepadidae) have to date been involved in karyotype analyses (Nakamura 1986) and studies of sperm and spermatid ultrastructure (Koike 1985; Buckland-Nicks & Chia 1986; Healy 1988; Hodgson *et al.* 1998).

Here we provide, to our knowledge, the first molecular phylogeny of the Neritopsina and discuss the history and degree of adaptive radiation within the group. The nucleotide sequences determined and analysed were from the large ribosomal molecule (28S rRNA) of eight neritopsine species and three outgroup species. The ingroup species were drawn from all the six extant families of the superorder recognized by Ponder (1998), i.e. Neritopsidae, Titiscaniidae, Hydrocenidae, Helicinidae, Phenacolepadidae and Neritidae, as well as from Neritiliidae, the family regarded as a distinct clade by recent morphological studies (Holthuis 1995; Kano & Kase 2000*a*,*b*, 2002).

2. MATERIAL AND METHODS

Table 1 lists the species analysed in this study together with relevant locality and habitat information of collected material. Although the Neritopsina is widely accepted as a distinct clade with a number of apomorphies in anatomy, embryonic development and sperm ultrastructure, much controversy exists concerning the group's relationships with the other major gastropod clades (e.g. Yonge 1947; Fretter 1965; Koike 1985; Salvini-Plawen & Haszprunar 1987; Haszprunar 1988; Fretter & Graham 1994; Van den Biggelaar & Haszprunar 1996; Harasewych *et al.* 1997; Ponder & Lindberg 1997; Sasaki 1998; Winnepenninckx *et al.* 1998). We therefore selected three species as outgroups to represent all the possible sister groups of the Neritopsina (table 1): i.e. Cocculiniformia (Cocculinidae), Vetigastropoda (Turbinidae) and Apogastropoda (Viviparidae).

Total DNA was isolated using a modification of the procedure of Doyle & Doyle (1987). Muscle tissues were homogenized in 300 µl of 2 × CTAB (cationic detergent hexadecyltrimethylammonium bromide) solution, 10 mg ml^{-1} of proteinase K, incubated at 60 °C for *ca*. 1 hour, extracted once with phenol/chloroform (v : v, 1 : 1) and precipitated with two volumes of ethanol. The DNA was briefly washed in 80% ethanol, air-dried for *ca*. 30 min, and dissolved in 50 µl of H_2O.

Approximately 1400 bp of partial sequences of 28S rRNA were amplified by PCR using primers 28Sna1: 5′-GACCC GTCTTGAAACACGGA-3′ and 28Sna2: 5′-AGCCAATCCT TATCCCGAAG-3′. PCR amplifications were performed using 35 cycles with the following conditions: denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s. The purified PCR products were cloned using the pGEM-T vector systems (Promega) and sequenced using automated sequencers (ABI PRISM 310 and/or HITACHI SQ5500). The sequence reactions were carried out using the Thermo sequenase pre-mixed cycle sequencing kit (Amersham) and Big Dye sequencing kit (ABI). A sequence of *ca*. 950 bp, corresponding to positions 5117–6181 in the published 28S rRNA sequence (*Mus musculus*, J00623), was sequenced in both strands.

Sequences were aligned in MegAlign (DNASTAR Inc.) using the default values of the parameters, and the alignments were corrected manually. Genetic distances among the sequences were obtained using the HKY85 distance model (Hasegawa *et al.* 1985). Gene trees were constructed using neighbour-joining (NJ) (Saitou & Nei 1987), maximum-likelihood (ML), and maximum-parsimony (MP) methods using Paup* (Swofford 1998). The reliability of the inferred phylogenies was tested by bootstrap resampling of the sequences (1000 replicates). A rate constancy of the 28S rRNA sequences among the taxa was tested using Felsenstein's (1993) test, a likelihood ratio test of unconstrained and clock-constrained trees.

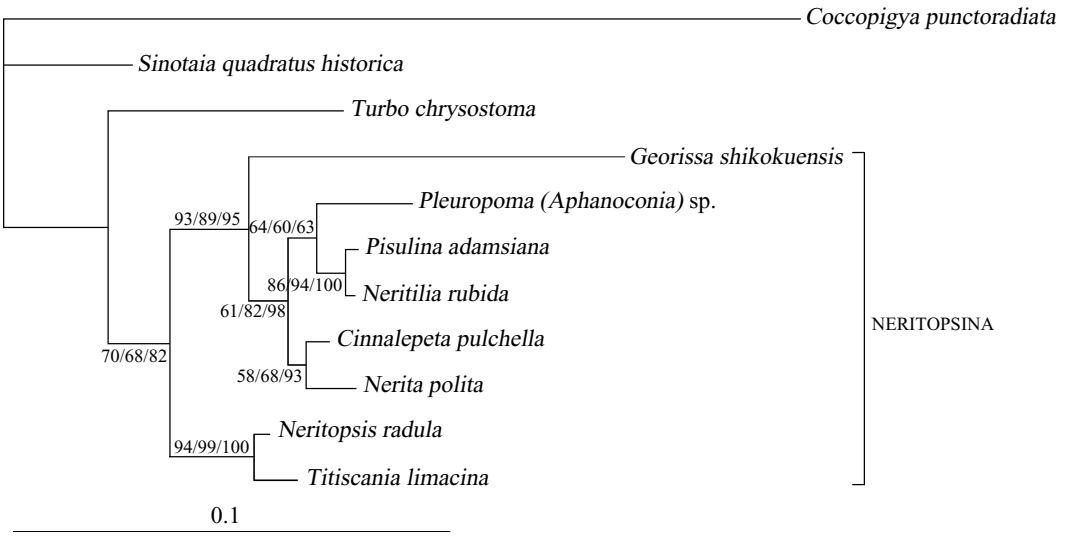

Figure 1. ML tree based on the 28S rRNA gene of 985 bp including gaps. The same topology was obtained in a single MP tree of 429 steps and an NJ tree using HKY85 distance (Hasegawa *et al.* 1985). The numbers at the branches are bootstrap values as a percentage of 1000 trials for ML, MP and NJ, from left to right.

3. RESULTS

All the sequences analysed here were newly determined and submitted to the DNA Data Bank of Japan (DDBJ) with the accession numbers listed in table 1. Ambiguously aligned sites that corresponded to 93–112 bp of *Neritopsis radula* were excluded from the analysis. Although some pairwise comparisons among taxa reflected little sequence divergence (less than 0.5%), most of the comparisons ranged from 2% to 10% divergence. Base frequencies averaged A, 25.5%; T, 19.8%; G, 31.2%; and C, 23.5%. The transition–transversion ratio was 0.68.

Phylogenetic relationships among the species of the Neritopsina were consistent among the NJ, ML and MP trees (figure 1). On the basis of the MP method, a single most parsimonious tree with 429 steps was generated; the consistency index for the tree was 0.81. The topology of the MP tree was consistent with that of NJ and ML (ln likelihood, −2167.9) trees. *Neritopsis radula* was phylogenetically the closest to *Titiscania limacina* among the neritopsine species examined in this study. Bootstrap values for this node were higher than 90%. *Neritilia rubida* and *Pisulina adamsiana* were consistently grouped together, and were placed into a clade with *Pleuropoma* (*Aphanoconia*) sp. *Nerita polita* and *Cinnalepeta pulchella* were also consistently grouped together. *Georissa shikokuensis* was phylogenetically closer to the clade of *Nerita* and *Neritilia* than that of *Neritopsis*.

The branch length of *G. shikokusensis* was considerably longer than that of other species, and a likelihood ratio test of unconstrained and clock-constrained trees rejected the null hypothesis of a constant rate of molecular evolution for the ingroup clade ($\chi^2 = 18.8$, $p < 0.01$). However, the null hypothesis of the rate constancy was not rejected when *G. shikokusensis* was excluded ($\chi^2 = 9.99$, $p > 0.05$). A rate constancy phylogeny of the ingroup clade except for *G. shikokusensis* is shown in figure 2.

4. DISCUSSION

(a) *Systematic implications*

The family Neritopsidae has the longest geological history among the extant neritopsines dating back to the Devonian or Silurian (Knight *et al.* 1960; Batten 1984; Bandel & Frýda 1999; Bandel 2000). The sole extant genus *Neritopsis* first appeared in the Middle Triassic (225 Myr ago) and is common in the Jurassic and Cretaceous shallow marine assemblages (Kase & Maeda 1980; Batten 1984). Today, the two extant species occur in such cryptic habitats as submarine caves and small pockets under deeply buried coral rubble in the tropical Indo-Pacific and Caribbean Sea. The present 28S rRNA analysis strongly supports the earliest divergence of the Neritopsidae as suggested by the fossil record (figure 1). Since the publication of a brief note on the external anatomy by Fischer (1875), the extant neritopsids had been poorly investigated until Holthuis's (1995) dissertation describing their vascular, excretory and reproductive organs. Holthuis claimed that the neritopsid open gonoduct is a primitive state, from which the closed duct in the other neritopsine families was derived.

Titiscania unequivocally represents the sister group of the *Neritopsis* in the present analysis. *Titiscania* remains a poorly known group with few specimens available for study (Ponder 1998), and there are many imperfections in former anatomical descriptions (e.g. Marcus & Marcus 1967; Houston 1990). Bergh (1890) established the superfamily Titiscanoidea and family Titiscaniidae for the sole species *T. limacina* by putting much emphasis on the shell-less, slug-like appearance. However, the radular and anatomical characteristics of *Titiscania* are almost identical to those of *Neritopsis*, except those modified in relation to the limacization (Kano 1999). *Titiscania* is in fact a very specialized offshoot of the Neritopsidae, and we therefore synonymize the younger name Titiscaniidae with Neritopsidae from the standpoint of cladistics (see below).

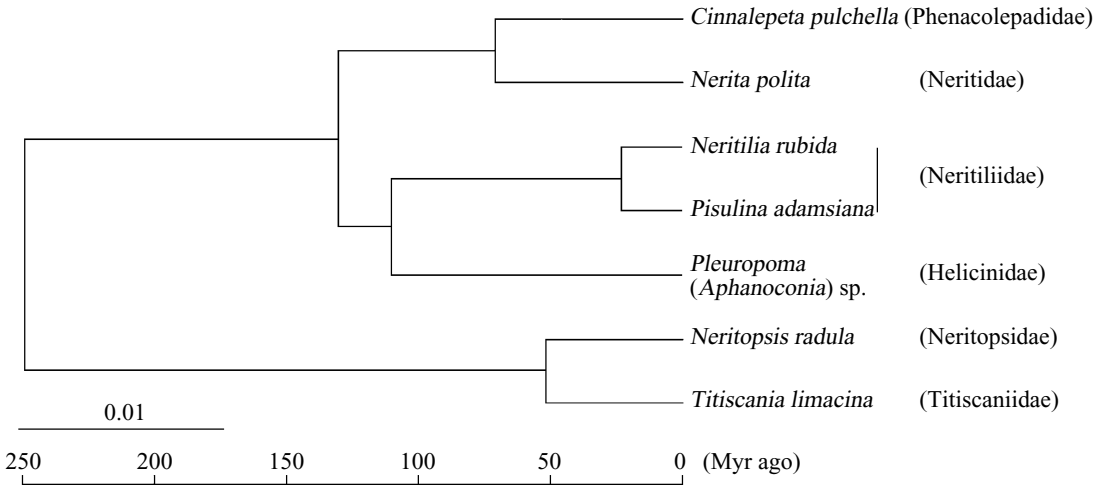

Figure 2. ML tree with estimated times of divergence based on molecular clock calibrations. *Georissa shikokusensis* (Hydrocenidae) is omitted due to its considerably high rate of evolution. To estimate the age of nodes, Bandel & Frýda's (1999) hypothesis derived from the fossil record is used herein: the first divergence among extant Neritopsina (i.e. Neritopsidae and others) is predetermined as 248 Myr ago. The earliest known fossil records for branches are as follows: 37–41 Myr ago for the Phenacolepadidae; 37–49 Myr ago for the Neritiliidae; and 89–93 Myr ago for the Helicinidae.

The 28S sequences indicate that the Hydrocenidae, Helicinidae, Neritiliidae, Phenacolepadidae and Neritidae constitute a clade sister to the Neritopsidae. Of the five families, the Hydrocenidae has a basal position; hydrocenids are minute, exclusively terrestrial snails, mainly inhabiting limestone outcrops in the tropics. The well-supported clade comprising the Helicinidae, Neritiliidae, Phenacolepadidae and Neritidae is divided into two subclades, one consisting of the Phenacolepadidae and Neritidae and the other of the Helicinidae and Neritiliidae. The close affinity of the former two families has also been suggested by a number of similarities in anatomical and spermatological traits (Fretter 1984; Holthuis 1995; Ponder & Lindberg 1997).

Neritids ('nerites') are the most common, familiar member of the superorder. They are found on intertidal and supratidal rocks and in mangroves on temperate to tropical coasts (e.g. *Nerita*), as well as in brackish-water and in freshwater (e.g. *Theodoxus*, *Clithon*, *Neritina* and *Septaria*). Species of *Smaragdia* feed on leaves of sea grasses and are the only non-cryptic inhabitants of shallow subtidal waters among the extant Neritopsina. A number of Mesozoic fossils have been assigned to the Neritidae, the oldest appearing in the Early Triassic (Knight *et al.* 1960; Tracey *et al.* 1993). However, this does not necessarily prove an independent history of the extant Neritidae since the Triassic. Rather, the hemispherical shell, typical of living neritids, appears to be a plesiomorphic condition from which the morphologically diverse shell forms of the latter group (all the families but Neritopsidae) have been derived.

Helicinidae plus Neritiliidae is the only clade supported by a low bootstrap value (less than 70%) in all the ML, MP and NJ trees. No definite morphological synapomorphy is known for the clade at present and its monophyletic nature is still uncertain. However, the female reproductive systems of the clade are somewhat similar to one another and distinctly different from the other neritopsines (vaginal and ootype openings widely separated; vaginal opening situated deep inside the pallial cavity) (Kano & Kase 2002). Moreover, the sperm ultrastructure of *Pisulina* (Neritiliidae) resembles that of *Waldemaria* (Helicinidae) rather than the Neritidae and Phenacolepadidae (J. Healy, personal communication). The helicinids are fully terrestrial, temperate to tropical snails, most of which inhabit leaf litter in moist forests, while some are arboreal. The distribution of modern helicinids ranges from East Asia and Australia to North and South America. In addition they have invaded remote islands in the Pacific Ocean.

From the Neritiliidae, both of the two extant genera (*Neritilia* and *Pisulina*) were selected as OTUs to test the monophyly of the family. The species of Neritiliidae had long been placed in three subfamilies of Neritidae based solely on shell shape (e.g. Baker 1923; Knight *et al.* 1960). However, recent anatomical and conchological studies have shown that the family is monophyletic and distant from the Neritidae (Holthuis 1995; Kano & Kase 2000*b*, 2002). The present molecular analysis strongly supports these morphological studies.

(b) *Character evolution*

Assuming that the 28S tree genuinely reflects evolutionary history, the morphological characters of shell, operculum, radula and heart, previously stressed in neritopsine classification (e.g. Baker 1923; Fretter 1965; Thompson 1980) seem to have only limited value in clade diagnosis. For example, limpets have evolved from coiled ancestors at least three times in Phenacolepadidae, Neritidae (*Septaria*) and Neritiliidae (an undescribed genus; figure 3); both concentric and paucispiral opercula occur in the Helicinidae, Neritiliidae and Neritidae; the radula lacks its central field in the primitive Neritopsidae as well as in the advanced Neritiliidae; and the diotocardian heart (retention of both right and left auricles) is found not only in the Neritopsidae but also in the Neritiliidae, Neritidae and Ceresinae of Helicinidae (e.g. Thompson 1980; Ponder 1998; Kano & Kase 2002).

Results of the present molecular analysis indicate that reproductive traits may be the only morphological charact-

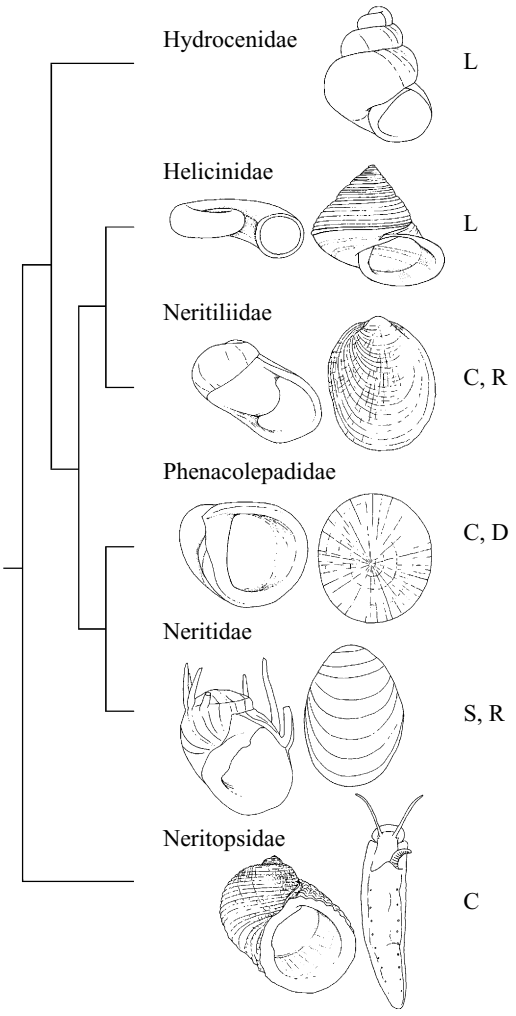

Figure 3. Hypothesis of phylogenetic relationships among extant neritopsine families, with reference to their present habitats. C, submarine caves and/or aquatic subterranean cavities; D, deep-sea hydrothermal vents and seeps; L, land (terrestrial and/or arboreal); R, riverine freshwater; S, shallow sea (non-cryptic).

ers that can be used in the diagnosis of all neritopsine clades. The male pallial gonoduct is composed of two ventrally open lamellae in the Neritopsidae (the plesiomorphic condition), while the lamellae have been fused ventrally and the duct is closed along its entire length in other neritopsines (the apomorphic condition). In the Neritopsidae and Hydrocenidae, females exhibit a monaulic reproductive system (a single gonopore acting as both vaginal and ootype openings: the plesiomorphic condition). In the other families, females are usually diaulic (vaginal and ootype have separate openings: the apomorphic condition; however, in some neritid genera there is the third, 'ductus enigmaticus' opening). The two openings are widely separated in the Helicinidae and Neritiliidae, whereas they adjoin in the clade Neritidae + Phenacolepadidae. The clade Neritidae + Phenacolepadidae is also diagnosed by the presence of a massive penis with a lateral groove (apomorphic) in males, unknown in the other neritopsines (see Bourne 1909, 1911; Thiele 1910; Andrews 1937; Holthuis 1995; Kano 1999; Kano & Kase 2002). The phylogeny of aquatic neritopsines (Holthuis 1995), based largely on the reproductive tract morphology, is congruent with the present molecular tree.

(c) *Invasion of the land*

More than nine gastropod clades have invaded the land and become fully terrestrial by evolving tolerance to desiccation in association with direct development in cleidoic eggs (Vermeij & Dudley 2000). Among the extant Neritopsina, the Hydrocenidae and Helicinidae are exclusively terrestrial, with a lung instead of a gill and intracapsular early development rather than a swimming veliger stage. Their dissimilarities in the reproductive tract and radula, however, led Bourne (1911) and Haszprunar (1988) to deduce that the two families are not closely related to each other and have independently colonized the land. Our molecular phylogeny shows that the Hydrocenidae and Helicinidae emerge as a paraphyletic group thus supporting the multiple colonization hypothesis (figure 3). Secondary marine invasion is very unlikely, as all the aquatic neritopsine families comprise planktotrophic species with characteristic, unquestionably homologous protoconchs (Bandel 1982; Bandel & Frýda 1999; Kano & Kase 2000*b*).

The Neritopsina achieved terrestrial invasion by the Carboniferous period. *Dawsonella meeki* from Illinois and Indiana, one of the oldest land snails known (Upper Carboniferous, *ca*. 300–315 Myr ago), has resorbed the coiling axis and inner whorls of the shell, leaving almost no internal traces of the previous whorls. Such an extensive resorption of the inner whorls has never been known outside the Neritopsina, and therefore *D. meeki* is certainly an early offshoot of the superorder (Solem 1983). Solem & Yochelson (1979) placed *D. meeki* in the Helicinidae, emphasizing its great congruence with the extant helicinids in shell morphology. *Dawsonella meeki* is sometimes placed in the family Dawsonellidae (e.g. Knight *et al.* 1960), presumably due to the enormous time gap between this species and the appearance of indisputable helicinids. Bourne (1909, 1911) considered the similarity in shell shape between *D. meeki* and Recent helicinids to be the result of convergence and concluded that both groups colonized the land independently.

We employed the molecular clock hypothesis for the present 28S sequences to estimate the timing of the helicinid divergence. Although a very high rate of evolution in *G. shikokusensis* precludes the estimation of the divergence date of the Hydrocenidae (figure 1), otherwise, the rate variation among ingroup clades is not so extensive and the rate constancy was not to be rejected for the sequences. To calculate the rates of molecular evolution, it is necessary to estimate the branching ages on the phylogeny. Here, we can test the above conflicting opinions concerning the true affinities of *D. meeki*. If this land snail were an early representative of the Helicinidae and the family had had an independent history since before the Late Carboniferous, the first divergence of the Neritopsina recognized in the present study should date back to the latest Precambrian, some 700 Myr ago. This is very unlikely, because the putatively oldest Gastropoda appeared in the Early Cambrian, 536–570 Myr ago (Runnegar 1996).

An alternative hypothesis, advanced by Bandel & Frýda (1999) on the basis of available fossil evidence, envisages the ancient Neritopsina splitting to form the Neritopsidae

and all remaining neritopsines, at some time near the Palaeozoic–Mesozoic boundary. We tentatively employed their hypothesis to calibrate the mutation rate: the first divergence among the extant Neritopsina was estimated as 248 Myr ago (figure 2). In this scheme, the above-mentioned earliest records of the families do not refute the estimated timing of the branching points, except that of *D. meeki*. The 'next oldest' helicinid *Dimorphoptychia* from the Upper Cretaceous (89–93 Myr ago) of Europe (Tracey *et al.* 1993) seems congruent with the estimated time of bifurcation of the Helicinidae in figure 2 (*ca.* 110 Myr ago). The fossil record of the family is rich and continuous since the Late Cretaceous onwards (Bishop 1980; Solem 1983). We therefore consider the Helicinidae originated in the Cretaceous and *D. meeki* (Dawsonellidae) derived from an ancient Neritopsina before the first bifurcation of the Neritopsidae and the others, and concur with Bourne (1909, 1911) that the resemblance between the Dawsonellidae and Helicinidae, both exhibiting a simple, conispiral shell form, is the result of convergence.

Little (1972) hypothesized a freshwater ancestry for the Helicinidae on the basis of physiological features. He found that helicinids are capable of producing dilute urine as do freshwater neritids, while terrestrial snails that supposedly evolved directly from a littoral ancestor (e.g. littorinoids) are unable to reabsorb salts from kidney fluid. Little (1990) later mentioned that the freshwater ancestry of the Helicinidae would be impossible, if the Palaeozoic *D. meeki* genuinely belongs to this family. This is the supposition rejected here. Indeed, the oldest freshwater neritopsine, *Mesoneritina morrisonensis* (currently included in the Neritidae), appeared in the Late Jurassic (Gray 1988), *ca.* 60 Myr before the first appearance of the Helicinidae. Moreover, the extinct Cretaceous family Deianiridae, which most closely resembles the Helicinidae in shell shape and possibly represents the sister taxon of the latter, has been recovered from coal swamp, fresh- and/or brackish-water deposits of Hungary (*ca.* 82–85 Myr ago) (Bandel & Riedel 1994). The occurrence of these non-marine aquatic neritopsines in the Cretaceous supports the freshwater, 'indirect' route to the land for the helicinids hypothesized by Little (1972, 1990). Interestingly, the same coal fauna of Hungary yield *Schwardtina cretacea*; this minute, aquatic species, provisionally placed in Neritidae, has teleoconch and protoconch morphology very similar to that of hydrocenids (see Bandel & Riedel 1994, plate 1). We therefore regard *S. cretacea* as the closest relative of the extant Hydrocenidae, whereas Tracey *et al.* (1993) listed the Late Miocene species as the oldest representatives of the family. Bandel (2000) considered that the present-day occurrence of the Hydrocenidae in the Old World as well as the New World, ranging from Australia to Asia and Europe, provides evidence for the Pangean origin of the family and therefore its history might extend back to the Early Mesozoic. The present molecular phylogeny indicates that the divergence of the family preceded that of the Helicinidae, but the timing of the terrestrial invasion by hydrocenids is uncertain. It seems safe, however, to say that hydrocenids might have invaded the land in the Mesozoic, possibly taking the freshwater route, as appears to be the case for the helicinids.

In conclusion, the present 28S data indicate that the Neritopsina has invaded the land and become fully terrestrial at least three times: dawsonellids in the Late Palaeozoic, helicinids in the Cretaceous, and hydrocenids, presumably in the Mesozoic. *Neritodryas* species of the Neritidae have also become somewhat terrestrial and even arboreal, being found in the coastal trees or on vegetation in the vicinity of fresh- and brackish-water (Little 1990; Cowie & Smith 2000), but they still retain planktotrophic early development and are thus not fully adapted to the land (Holthuis 1995). In contrast to the terrestrial invasions, the invasions of fresh- and brackish water constitute a more significant element in the evolutionary history of the Neritopsina. At least three freshwater and/or brackish lines (*Mesoneritina*, *Deianira* and *Schwardtina*) seem to have arisen in the Mesozoic. In the Cenozoic, the Neritidae and Neritiliidae each gave rise to at least one freshwater line. Based on her extensive anatomical and phylogenetic works, Holthuis (1995) concluded that there are at least 12 shifts between marine, brackish and freshwater in the Neritidae, and that the origin of an osmoregulatory kidney in brackish waters is followed by five or six evolutionary colonization shifts into freshwater.

(d) *Colonization of deep-sea vents and seeps*

In addition to terrestrial and freshwater invasions, neritopsines also radiated into deep-sea, chemosynthetically nourished communities. The Phenacolepadidae is a group of limpets inhabiting dysoxic, sulphide-rich environments in warm-temperate to tropical shallow seas and found on the under-surface of deeply embedded stones and decaying wood in soft sediments (Fretter 1984; Warén & Bouchet 2001) where they probably feed on chemosynthetic bacteria. These subterranean inhabitants have extremely long cephalic tentacles and numerous contractile tentacles along the mantle edge, compensating for loss of sight (Fretter 1984). Their blood is red as they have erythrocytes to increase the capacity of blood to transport oxygen (Fretter 1984; Sasaki 1998). Warén & Bouchet (1993, 2001) considered that an ability of the larvae to recognize chemosynthetically nourished dysoxic biotopes, as well as the use of haemoglobin as a respiratory pigment, was a starting point of the evolution of taxa restricted to vents and seeps. Species of *Shinkailepas* and *Olgasolaris* (Phenacolepadidae) have so far been collected only from hydrothermal vents and mineral seeps (Okutani *et al.* 1989; Beck 1992; Warén & Bouchet 1993, 2001). They are examples of 'a few vent species closely related to (and presumably derived from) shallow-water genera' (Van Dover 2000, p. 314).

It is interesting to note that *Bathynerita naticoidea*, from deep-sea oil and gas seeps in the Gulf of Mexico, currently placed in the Neritidae, is most probably a member of Phenacolepadidae, regardless of its coiled, neritid-like shell. Its close affinity to phenacolepadids rather than to neritids has been confirmed by detailed anatomical and spermatological studies (Holthuis 1995; Hodgson *et al.* 1998). The transition from snails with plesiomorphic, hemispherical shells to apomorphic limpets was most probably achieved after the establishment of the family (see above), therefore indicating *B. naticoidea* as the archetype of the Phenacolepadidae. The neritid-like radula and

apparent absence of erythrocytes (Warén & Bouchet 2001) also favour this view.

We present two possible explanations for the basal position of *Bathynerita*. First, the coiled and uncoiled groups have independently invaded the deep-sea communities. Second, the family incorporated into the vent and seep communities has secondarily colonized the ecologically similar, shallow-water dysoxic subterranean environments. The shallow-water limpets and deep-sea snails appeared almost simultaneously in the fossil record. The oldest phenacolepadid limpet *Plesiothyreus parmophoroides* is from Middle Eocene shallow-water deposits of France (37–41 Myr ago) (Knight *et al.* 1960; Tracey *et al.* 1993). By contrast, *Thalassonerita*, the fossil genus closely resembling *Bathynerita*, has been recorded from the bathyal cold-seep deposits of the Middle Eocene in western Washington (*T.*? *eocenica*) and the Late Miocene in Italy (*T. megastoma*) (Squires & Goedert 1996). Whether or not multiple colonization occurred, it seems safe to say that phenacolepadids have been components of the deep-sea, chemosynthetically nourished communities since the Late Cretaceous or at least the Early Cenozoic (figure 2).

Based on partial 28S rRNA sequences, McArthur & Koop (1999) proposed a fundamentally different hypothesis on the neritopsine phylogeny ((*Bathynerita* + *Shinkailepas*) (*Theodoxus* (*Nerita* + *Olgasolaris*))). However, the non-monophyly of phenacolepadids is most unlikely, considering their close resemblance in morphology (Fretter 1984; Beck 1992; Holthuis 1995), and none of the neritopsine subclades in McArthur & Koop (1999) is robustly supported. As is clear in the results of Colgan *et al.* (2000) and the present study, too low a substitution rate of the 28S makes this gene difficult to use for reconstruction of phylogeny within certain gastropod families. A better understanding of the history of deep-sea invasion requires detailed reconstruction of phenacolepadid phylogeny.

(e) *Refugees in submarine caves*

Leaving aside the Phenacolepadidae, the adoption of a cryptic habit is not rare among the Neritopsina (figure 3). The archaic family Neritopsidae particularly flourished in the Late Palaeozoic and Early Mesozoic, but only one genus (*Neritopsis*) is known in post-Cretaceous times (Batten 1984). Today, *Neritopsis* is represented by two species, which exclusively inhabit submarine caves and similar cryptic voids in shallow seas (Kase & Hayami 1992; Warén & Bouchet 1993; Holthuis 1995). Submarine caves are generally characterized by low predation pressure and offer suitable refuges to archaic organisms (Iliffe *et al.* 1984; Hayami & Kase 1996; Iliffe 2000). Shells of *Neritopsis* are quite common in Mesozoic shallow-water, soft bottoms, sometimes accompanied by an operculum preserved *in situ* (Kase & Maeda 1980; Das *et al.* 1999). This indicates that originally *Neritopsis* was non-cryptic. We are tempted to assume that *Neritopsis* began to exploit the cryptic habitats by the Middle Cenozoic. Since the beginning of the Cenozoic the numbers of species and individuals of *Neritopsis* greatly decrease. P. Lozouet (personal communication) found an unidentified *Neritopsis* species from an Oligocene molluscan assemblage in France, which includes many genera common in the Recent submarine caves.

Another neritopsid species *T. limacina* (herein reallocated from its own family Titiscaniidae) is found only in small interstices under deeply buried coral rubble or stones. The back of this slug bears defensive glands that discharge white threads when the animal is disturbed (Bergh 1890; Marcus & Marcus 1967); these must compensate for the lack of a shell, a very effective armour against predation. The slug has an advantage over the shelled neritopsids in small shelters. Likharev & Wiktor (1980, pp. 79–83) supposed that evidently polyphyletic, terrestrial slugs have lost their shell several times in order to utilize protective spaces provided by rock fissures, pockets under stones and earthworm tunnel tracks. The ancestor of *T. limacina*, diverged from a certain neritopsid (possibly *Neritopsis*) species, might find the shelter and undergo complete limacization with loss of the shell. No fossil of *Titiscania* is known, as is usual with shell-less slugs, but there is possible concordance between the above-mentioned timing of cryptic habitat colonization by neritopsids and the postulated divergence of *Titiscania* (figure 2).

The Neritiliidae is another clade utilizing submarine caves as refuges. *Pisulina* and several undescribed genera exclusively inhabit caves (Kano & Kase 2000*b*, 2002). The earliest fossil record of this family is much younger than the Neritopsidae; the oldest neritiliid occurring in the Eocene of France (P. Lozouet, personal communication). However, we believe that the younger history of the Neritiliidae may be due to an incomplete documentation of fossil species. Neritiliidae are so similar to the Neritidae in teleoconch morphology that it is possible (and even likely) that pre-Eocene neritiliids have been dismissed as neritids. Detailed study on the protoconch and shell microstructure may clarify the distinction between the two groups (Kano & Kase 2000*b*).

The Neritiliidae occupy an unusual range of habitats. *Neritilia* lives in freshwater streams, brackish estuaries, groundwaters and anchialine waters (bodies of haline water with more or less extensive subterranean connection to the sea, and showing noticeable marine as well as terrestrial influences (Stock *et al.* 1986; Kano *et al.* 2001; Kano & Kase 2002; Sasaki & Ishikawa 2002)). Curiously, no marine species is known outside caves. A cryptic habit has also been suggested for the Miocene genus *Pisulinella* (Kano & Kase 2000*a*) and an undescribed genus from the Oligocene (P. Lozouet, personal communication). Kano & Kase (2002) found an open pit eye without a vitreous body in *Pisulina* and *Neritilia*: a condition hitherto unknown among the subclass Orthogastropoda. If the eyes of *Pisulina* and *Neritilia* are degenerative in origin (apomorphic condition), the loss of the cornea and vitreous body almost certainly took place in a common ancestral species that lived in gloomy-to-totally dark, cryptic habitats (Kano & Kase 2002). In this scheme, the invasion of the surface, riverine habitat by *Neritilia* is most convincingly explained by the presence of underground corridors, where a number of neritiliid species are known to live (Kano *et al.* 2001). More detailed molecular phylogeny, in combination with morphological study, must give us insight into the history of colonization in the Neritiliidae.

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