

Escalation and trophic specialization drive adaptive radiation of freshwater gastropods in ancient lakes on Sulawesi, Indonesia

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Species flocks in ancient lakes have long been appreciated as ideal model systems for the study of speciation and adaptive processes. We here present data from a new invertebrate model system with intrinsic parameters distinct from those of other documented radiations. The ancient lakes on Sulawesi harbour an endemic species flock of at least 33 species of viviparous snails. Molecular data reveal multiple independent colonizations of the lakes by riverine ancestors. In each colonizing clade, parallel evolution of conspicuous shell morphologies, followed by a differentiation of trophic morphology and the development of habitat specificity can be observed. Extensive shell crushing experiments and strong dentition of the chelae observed in some lacustrine crab species suggest that coevolution with crabs, i.e. escalation, is the most likely cause of initial shell divergence. By contrast, repeated parallel evolution in radula morphology indicates that speciation within lineages is driven by divergent adaptation to different resources among sympatric taxa. The inclusion of coevolutionary processes is unique in this system compared with diversification models developed for vertebrate radiations.

Keywords: ancient lakes; freshwater gastropods; molecular phylogeny; parallel evolution; escalation; *Tylomelania*

1. INTRODUCTION

Speciation and adaptation are central tenets of evolutionary biology. Species flocks such as the Galapagos finches (Sato *et al.* 2001), the Hawaiian drosophilids (DeSalle 1992) and the East African cichlids (Meyer *et al.* 1994) exemplify the results of both processes. It is generally assumed that species flocks arise through the process of adaptive radiation (Rossiter & Kawanabe 2000), the rapid divergence of a lineage into phenotypically and ecologically distinct species (Schluter 2000) by ecological speciation. Adaptive radiations are usually associated with insular habitats, and ancient lakes stand out for their especially high diversities of endemic organisms (Rossiter & Kawanabe 2000).

Extensive study of the cichlid species flocks from the East African lakes has led to the recent development of models suggesting that initial bursts of trophic specialization after colonization of a lake are often followed by assortative mating and sexual selection, leading to extensive speciation (Sturmbauer 1998; Albertson *et al.* 1999; Danley & Kocher 2001). While fish species flocks continue to contribute to our knowledge of speciation and adaptation, the endemic invertebrates present in almost every ancient lake have been largely neglected (Rossiter & Kawanabe 2000). Because of their mode of reproduction,

limnic gastropods have very different dispersal abilities (Cohen & Johnston 1987) and are, unlike cichlid fishes, less prone to sexual selection (but see Rolán-Alvarez *et al.* 1995).

Two ancient lake systems in the central mountains of the Indonesian island of Sulawesi (figure 1) harbour an endemic species flock of viviparous freshwater gastropods (Caenogastropoda: Cerithioidea: Pachychilidae). The two ancient lake systems on Sulawesi are solitary Lake Poso and, ca. 80 km southeast, the five lakes of the Malili lake system, which are all connected by rivers (figure 1). Lake Matano, at ca. 590 m the deepest lake of Indonesia, is a typical *graben*-lake (Haffner *et al.* 2001). Lake Poso and the Malili lakes are situated in two different faults and have never been connected (Wilson & Moss 1999). All major lakes are ca. 1–2 Myr old, according to geological estimates (R. Hall, personal communication).

Discovered and first described a century ago by the two Swiss naturalists and ethnologists Paul and Fritz Sarasin (Sarasin & Sarasin 1898), the 33 endemic species—today attributed by current taxonomic studies (von Rintelen 2003) to the genus *Tylomelania* Sarasin & Sarasin, 1897—have long escaped detailed study (Marwoto 1997). These gastropods have been occasionally cited as an example of intralacustrine adaptive radiation in ancient lakes (Brooks 1950; Davis 1982), but any ecological or biological data have been lacking. In this paper, we use the endemic gastropods of these lakes to test pivotal assumptions of adaptive radiation such as monophyly of lake species

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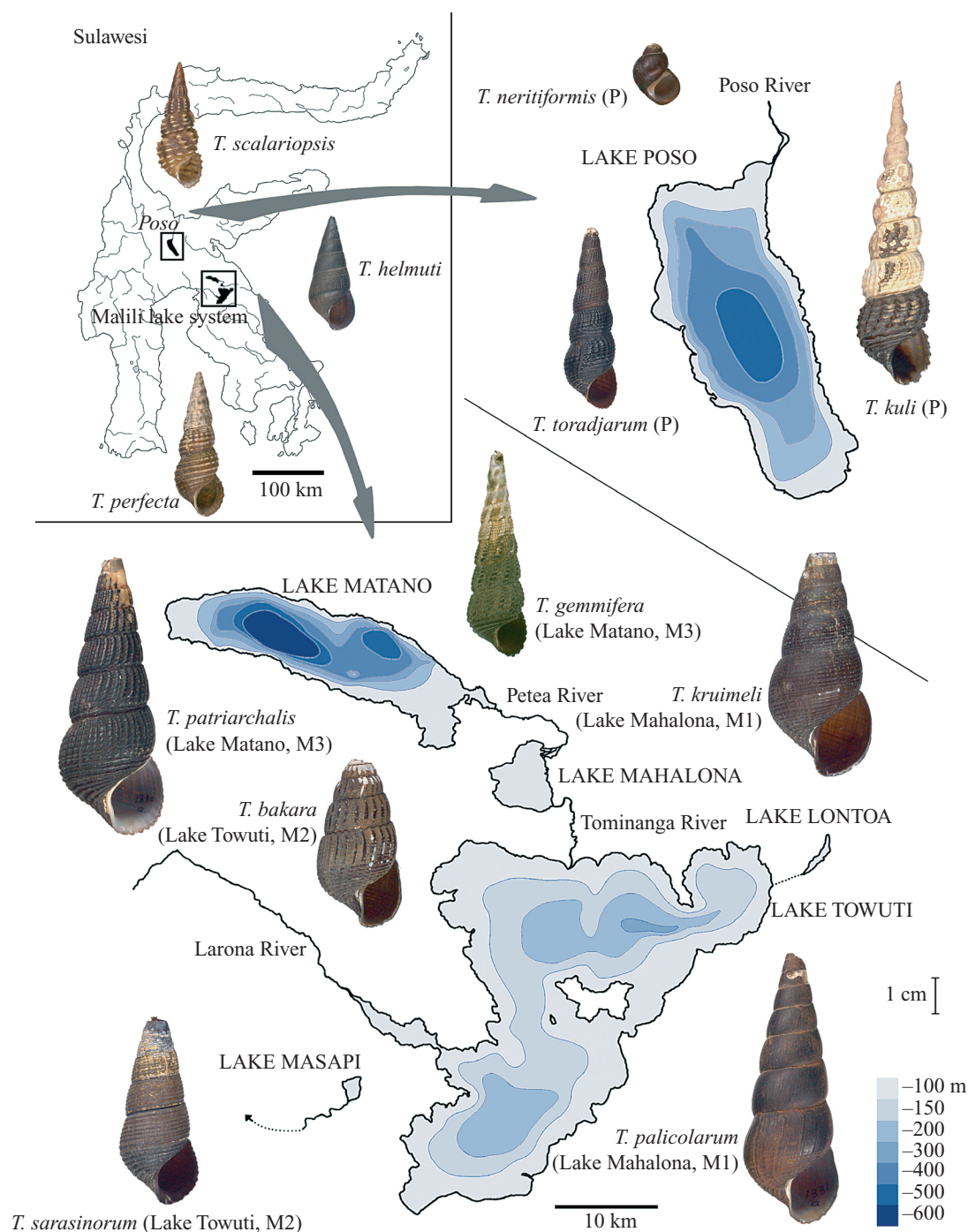


Figure 1. Sulawesi, the ancient lakes and characteristic gastropod species. The letters in parentheses after the species names refer to their respective clade (see figure 3*a,b*).

flocks, and investigate factors that may be promoting intra-lacustrine diversification.

2. MATERIAL AND METHODS

(a) *Material*

Material was collected from many of sites both in the lakes and in rivers (figure 1) by M. G. and T. v. R. in 1999 and 2000, and all samples were fixed and preserved in 70–96% ethanol. Basic ecological data such as substrate (hard substrate (rock and wood) versus soft substrate (mud or sand)) and depth were recorded. All voucher material is deposited in the Malacological Department,

Museum of Natural History, Berlin. The accession numbers (prefix ‘ZMB’) are provided in figure 2 after species labels.

(b) *Molecular methods*

Two mitochondrial gene fragments, 646 bp of cytochrome oxidase subunit 1 (COI) ($n = 181$ specimens) and 834 bp of 16S ($n = 191$ specimens), from at least one population of almost all lacustrine ($n = 29$) and most riverine species ($n = 8$), were amplified and sequenced on an ABI 377 DNA sequencer using universal primers (LCO1490 (Folmer *et al.* 1994) and HCO2198var. 5'-TAWACTTCTGGGTGKCCAAARAAAT-3' for COI; 16SF (Wilson *et al.* 2004) 5'-CCGCACTAGTGATAGCTAGTTTC-3'

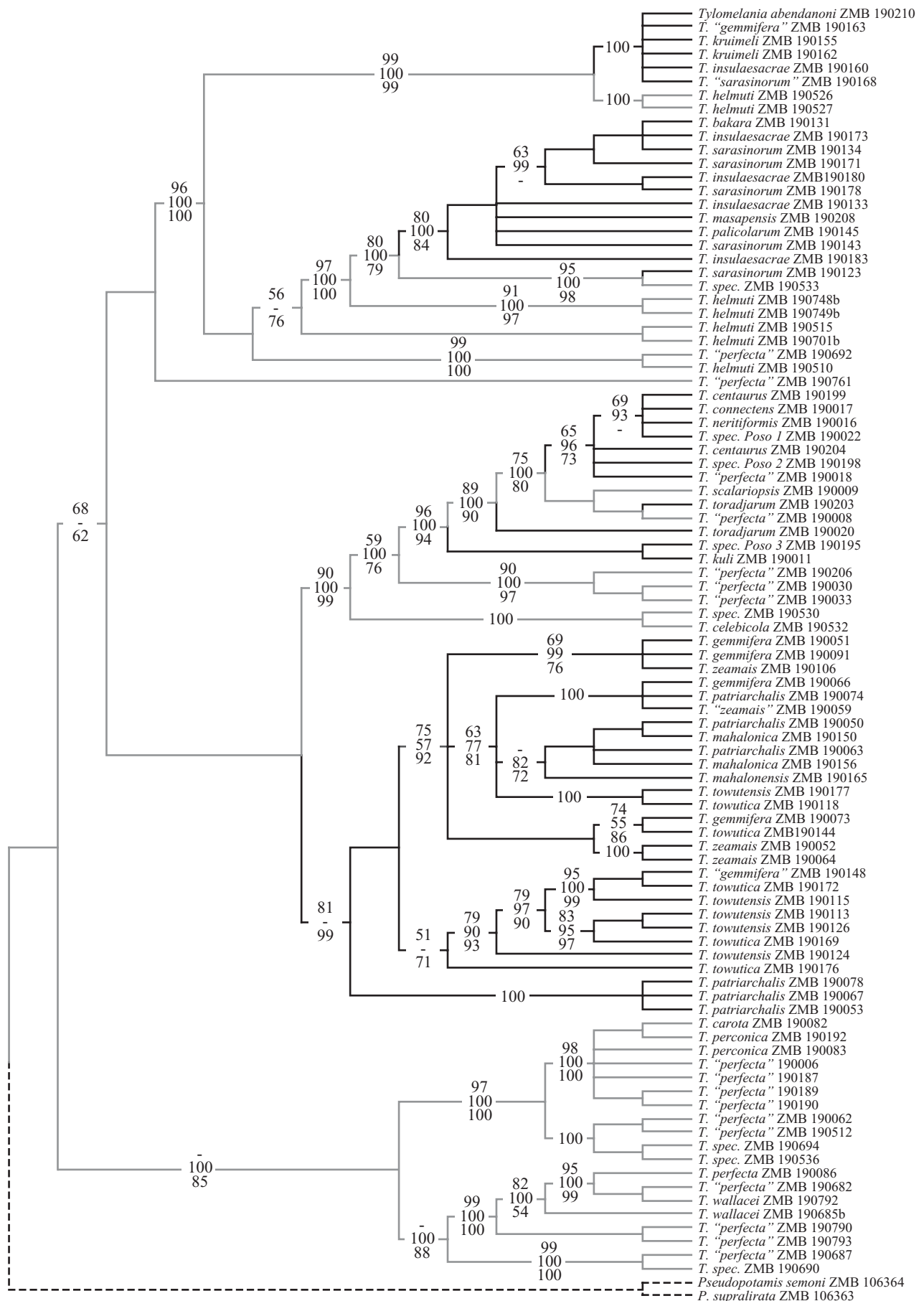


Figure 2. Maximum parsimony strict consensus of 13 432 equally most parsimonious trees (1276 steps). The numbers on the branches are, from top: MP bootstrap values; Bayesian posterior probabilities; and NJ bootstrap values. Black lines, lacustrine taxa; grey lines, fluvial taxa; hatched lines, outgroup.

and H3059var. (Palumbi 1996) 5'-CCGGTYTGAAGTCA-GATCATGT-3' for 16S). The alignment was subsequently reduced to 95 sequences to make it computationally feasible for the phylogenetic analyses through the elimination of identical or very similar haplotypes of the same species. All sequences have been deposited in GenBank, accession numbers AY242953–242970 and AY311821–312053.

(c) *Phylogenetic analyses*

The orthologous DNA sequences obtained were aligned, using default settings, by CLUSTALW, v. 1.81 (Thompson *et al.* 1994) and optimized by eye. The aligned sequence sets of COI (646 bp) and 16S (852 bp) were combined into a single alignment (1498 bp). While a parsimony-based incongruence length difference test rejected data homogeneity ($p < 0.001$), we concatenated sequences in concordance with a total evidence approach (Barker & Lutzoni 2002). For comparison, trees based on analysis of the separate datasets are provided in electronic Appendix A.

Two species of the North Australian pachychilid *Pseudopotamius*, recently proposed as a sister taxon to *Tylomelania* (Glaubrecht & von Rintelen 2003), were chosen as an outgroup to root the phylogeny. Neighbour-joining (NJ) distance and maximum parsimony (MP) analyses were performed using PAUP, v. 4.0b010 (Swofford 1998), with indels coded as a fifth base where applicable. Bootstrapped NJ analyses (1000 replicates) were performed under the HKY85 + Γ + I model of substitution with parameters estimated by MRMODELTEST, v. 1.1 (Nylander 2002). Parsimony analyses were performed with a full heuristic search (100 bootstrap replicates) using random addition (10 replicates), the tree bisection–reconnection branch swapping algorithm and the MULPARS option. Phylogenetic relationships were also approximated following a Bayesian method of phylogenetic inference as implemented by MRBAYES, v. 2.01 (Huelsenbeck & Ronquist 2001). Posterior probabilities of phylogenetic trees were estimated by a 1 000 000 generation Metropolis-coupled Markov chain Monte-Carlo (four chains, chain temperature = 0.2) under the HKY model of substitution, with parameters as estimated by MRMODELTEST (see above). A 50% majority-rule consensus tree was constructed following a 500 000 generation burn-in to allow likelihood values to reach stationarity. Character mapping was carried out with MACCLADE, v. 4.0 (Maddison & Maddison 2000). To investigate the hypothesis of a single lacustrine colonization, a likelihood-based Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa 1999) was used (10 000 RELI bootstraps), comparing the maximum likelihood topology to a topology where lake taxa were constrained as a monophyletic group.

(d) *Morphology*

Scanning electron microscopy was used to study the radula of individuals ($n = 1$ –12) from every sampled population. Shell thickness was measured with an electronic calliper to 0.01 mm ($n = 3$ specimens per sample). The occurrence of shell repair was recorded for $n = 514$ specimens of lacustrine species ($n = 8$) and $n = 126$ specimens of riverine taxa ($n = 3$).

(e) *Shell crushing experiments*

Shells ($n = 117$ specimens from 12 populations of three riverine and eight lacustrine species) were crushed with Zwick and Instron pressure resistance test devices (500 and 5000 N maximum force, respectively) at the Bundesanstalt für Materialforschung und -prüfung (Berlin). The maximum force needed to initiate cracking of the shell was measured. Shells were positioned with the apertural plane parallel to the force exerting surface. Body whorl height used was as an estimator of shell height in the graphic

representation of the crushing experiment, as almost all shells have corroded upper whorls to an individually varying degree.

3. RESULTS AND DISCUSSION

(a) *Molecular phylogeny and lacustrine colonization*

The molecular phylogeny based on parsimony (figures 2 and 3*b–d*) and NJ analyses (figure 3*a*) reveals four strongly supported clades within the lakes, i.e. one clade in Lake Poso (P) and three clades in the Malili system (M1–M3). Riverine taxa are sister groups to three lacustrine clades, the Poso (P) and two of the Malili clades (M1 and M2). The tree topology is most parsimoniously interpreted by assuming an independent colonization of the lakes by all four lineages (figure 3*a*) and the hypothesis of a single lake colonization is rejected (SH test; $p < 0.001$). Although a separate invasion of Lake Poso and the Malili lakes is an expected result given that the two lake systems were never connected, it is surprising that colonization took place independently from different ancestral lineages in the three major lakes of the Malili system, which are directly connected by rivers. Two clades, M1 ($n = 5$ species, Lakes Mahalona and Lontoa) and M2 ($n = 5$ species, Lake Towuti and Masapi), are still primarily restricted to the lake first colonized in the Malili system (figure 3*b*). By contrast, clade M3 ($n = 9$ species) spread widely and is now present in all major lakes of the system. In Lake Poso, lacustrine species ($n = 8$) appear to have recolonized riverine habitats in two cases (asterisks in figure 3).

(b) *Shell morphology in riverine and lacustrine clades*

Dramatic changes in shell morphology are associated with lake colonization (figure 1). Shell morphology within each lacustrine clade is similar and clade-specific, providing additional support for the independent colonization of the Malili lake system by clades M1–M3. M1 and M2 species generally have smooth or spiral-ribbed shells (e.g. *Tylomelania palicularum* and *T. sarasinorum* in figure 1), whereas all M3 species have shells with axial ribs (e.g. *T. gemmifera* and *T. patriarchalis* in figure 1). Species can be distinguished by their characteristic shells, although intraspecific variability is rather high (von Rintelen & Glaubrecht 2003). In each lacustrine clade, convergent evolution of thicker shells relative to riverine species occurred in almost all cases (figure 3*c*). All Sulawesi pachychilids have three crosslamellar shell layers and shell strength is highly significantly correlated with shell thickness (figure 4*c,d*; $r = 0.779$, $p < 0.001$). Thus, shell thickness can be regarded as indicative of shell strength and is used here as an estimator of resistance to crab predation (see Boulding & LaBarbera (1986) for limitations of this procedure). The various shell sculptures present in *Tylomelania* (see above, figure 1) are also believed to influence shell vulnerability (Vermeij 1993). A relatively high proportion of specimens from the lakes have repair scars (50.6% versus 18.8% in fluvial species; figure 4*a*), and the frequency of shell repair is significantly higher in lacustrine than in riverine taxa ($t = 4.352$, $p < 0.05$). These findings coincide with the occurrence of one species of molluscivorous crabs in each of the lakes (Sundatelphusidae and Parathelphusidae; C. Schubart, unpublished data), which possess pronounced dentation on their chelae, enabling them to crack shells (figure 4*b*). This dentation is lacking in the other two or three

lacustrine crab species per lake and the widespread riverine taxa (C. Schubart, unpublished data). The position of the repair marks on the shell (figure 4a) parallels those inflicted by some marine crabs which attempt to 'peel' the snail by starting to chip off shell pieces from the aperture, and suggests a similar behaviour in the Sulawesi crabs.

(c) Variation in trophic (radula) morphology

The second major character group changing strikingly in the lakes is radula morphology, which is a pivotal part of the trophic system in gastropods and indicative of food and substrate preferences (Hawkins *et al.* 1989; Taylor & Lewis 1995). The molluscan radula is generally considered a conservative character with little variation at the species level (but see Padilla 1998; Reid 2000). Among the Sulawesi pachychilids, most riverine species possess identical radulae (figures 3d and 5a,b; *T. perfecta*). By contrast, nine different species-specific radula morphologies distinguished by the shape and relative size of their denticulation are present within the lake clades (figures 3d and 5b–k), with three to six morphotypes found in each clade. Trophic morphology and substrate are highly correlated in all clades. Soft substrate species have identical or very similar radulae to those found in riverine taxa (figure 5b,c), whereas hard substrate taxa often have strongly enlarged teeth (figure 5e–k; Spearman's ρ : 0.708, $p < 0.01$). The tight correlation between enlargement of radula denticles and hard substrate is further supported by the parallel occurrence of accordingly modified radulae in hard substrate dwellers, in both ancient lake systems and in the only constantly rock-dwelling riverine taxa found so far on Sulawesi (see basal clade in figure 3d). These observations suggest a functional role for the differences found, although a detailed understanding of the underlying mechanisms requires further investigation. The molecular phylogeny reveals that in all but one clade (M1) distinct, i.e. 'lacustrine' (figure 5c–k) as opposed to typical 'riverine' (figure 5b) radula morphologies evolved after colonization of the lakes. At the same time, a considerable number of lake species have retained a typically 'riverine' radula morphology.

The strict correlation between lacustrine colonization and gastropod shell strength is not reflected in the change of trophic morphology. This might indicate concordance with expectations from models derived from the cichlid radiations in East Africa (Sturmbauer 1998; Danley & Kocher 2001), where trophic specialization is expected to occur shortly after habitat preferences are established. By contrast, a considerably different pattern has been observed in the gastropod species of ancient Lake Tanganyika, where lineage diversification and the evolution of morphological disparity appear to pre-date lake formation (Wilson *et al.* 2004).

(d) Escalation

The divergent patterns of variation in shell and trophic morphology suggest that different factors may have been involved in their evolution. Escalation, i.e. the coevolution of predators and prey (Vermeij 1987, 1994), has repeatedly been discussed as a factor in the evolution of the thalassoid, i.e. marine-like and highly bizarre lacustrine shell morphologies found in Lake Tanganyika snail species (Vermeij &

Covich 1978; West *et al.* 1991; West & Cohen 1996; but see Wilson *et al.* 2004). In both Lake Poso and the Malili system, our data on lacustrine gastropod shell strength, structure and repair, in combination with the occurrence of large molluscivorous crabs, suggests that escalation is a driving factor in initial shell divergence upon colonization of the lakes.

(e) Speciation patterns

The finding of largely species-specific radula types and the correlation between trophic morphology and substrate in *Tylomelania*, as well as marked niche differences in species (von Rintelen & Glaubrecht 2003), suggest a strong role for ecological factors in speciation in these snails. In gastropods, studies on intertidal marine species of *Littorina* have indicated differentiation across ecotones (Johannesson *et al.* 1995; Kyle & Boulding 1998; Wilding *et al.* 2001) and assortative mating between morphs (Saur 1990). The limnic gastropods of Sulawesi offer excellent opportunities to test the importance of these factors in freshwater.

As an alternative to ecological speciation (Schluter 2001), allopatric speciation with initial random differentiation of populations and subsequent competition in sympatry might explain the observed pattern. Allopatric speciation is predicted within clade M3 (figure 2a,b), where three lineages are present. Two occur sympatrically in Lake Matano and Lake Mahalona (M3.1, M3.3), whereas one is almost exclusively confined to Lake Towuti (M3.2) and largely allopatric to the other two. However, independent parallel evolution of hard and soft substrate dwellers with associated trophic traits (and size differences) occurred in two of the three lineages (M3.1 and M3.2; figure 3d), suggesting an important role for ecology-driven differentiation, even if the initial phase of allopatric speciation has not been associated with ecological divergence. A particularly striking case of parallel evolution is represented by two shallow water mud dwellers in Lake Poso (*T. kuli*) and the Malili system (*T. gemmifera*). Both species have essentially the same radula type (figure 5c, asterisks) and similar, turreted shells with prominent axial ribs (figure 1). Although similar patterns might be suspected for several other species, a lack of resolution at the species level within all lake clades probably caused by incomplete mitochondrial lineage sorting, as a result of rapid speciation or secondary introgression, prohibits a more detailed discussion of convergent evolution for the time being.

(f) Concluding remarks

The evolution of stronger shells in each lineage after lake colonization appears to have been prerequisite for the subsequent radiation within each clade. Considering the complex pattern found in the Sulawesi system involving both allopatric speciation and the parallel development of habitat specificity associated with changes in trophic morphology, we suspect that both geography and ecology have been driving speciation. Simple models of intralacustrine gastropod radiation proposed for snails (Cohen & Johnston 1987; Johnston & Cohen 1987; Michel 2000) focusing on only one intrinsic factor, often fail to account for the observed patterns, as highlighted by Wilson *et al.* (2004) for the Lake Tanganyika species flock.

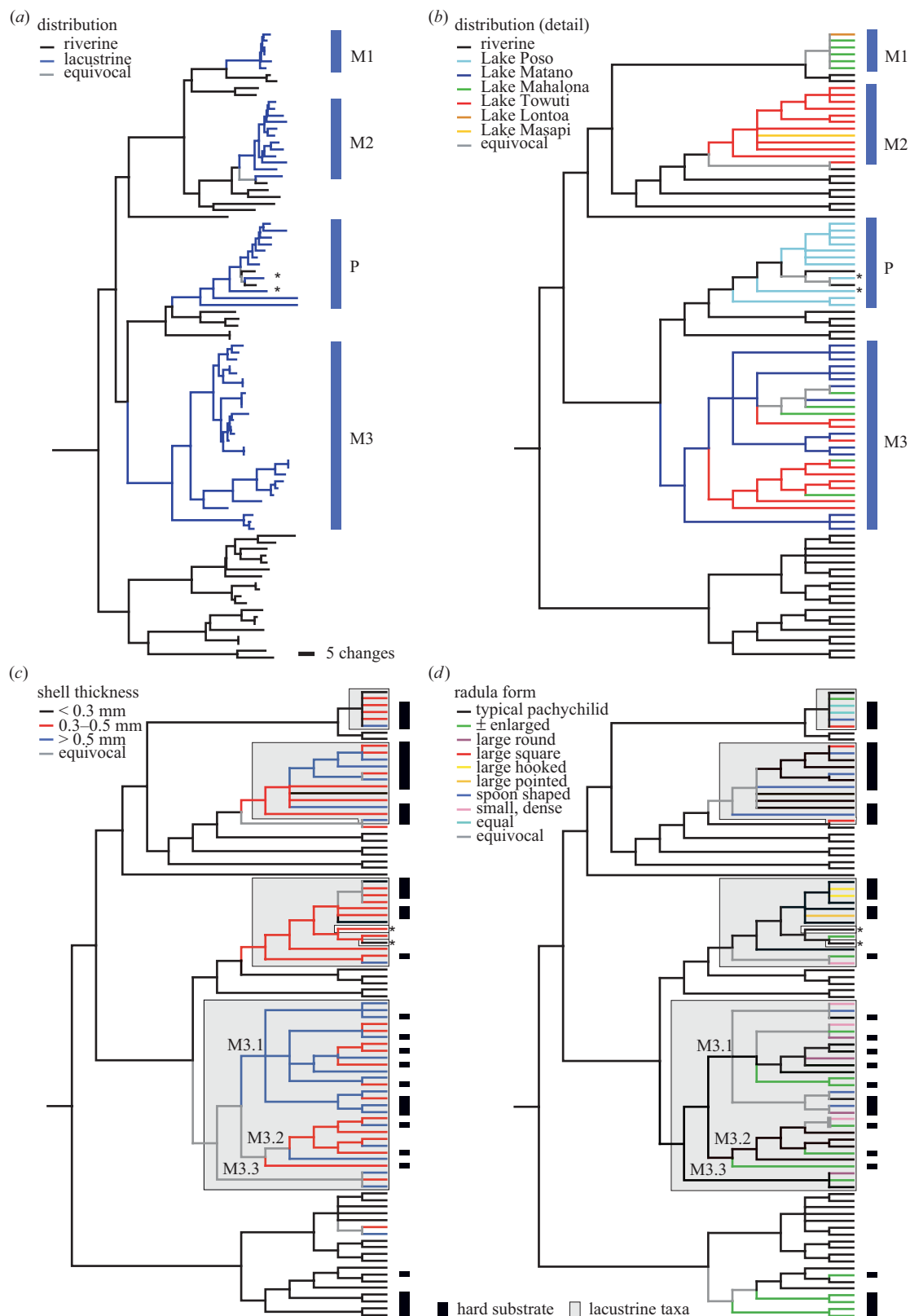


Figure 3. Molecular phylogeny and character distribution. (a) NJ phylogram and (b–d) MP strict consensus tree (compare figure 2). (a) Lake colonization. Black lines indicate riverine taxa; blue lines indicate lacustrine clades; asterisks mark riverine taxa in clade P; grey lines indicate equivocal clades. (b) Lake colonization in detail. Black lines, riverine; light blue lines, Lake Poso; dark blue lines, Lake Matano; green lines, Lake Mahalona; bright red lines, Lake Towuti; brown–red lines, Lake Lontoa; orange lines, Lake Masapi; grey lines, equivocal. (c) Shell thickness. Black lines, less than 0.3 mm; red lines, 0.3–0.5 mm; blue lines, more than 0.5 mm; grey lines, equivocal. (d) Radula form. Black lines, typical pachychilid; green lines, \pm enlarged denticles; brown–red lines, large round denticles; bright red lines, large square denticles; yellow lines, large hooked denticles; orange lines, large pointed denticles; blue lines, spoon shaped denticles; pink lines, small dense denticles; turquoise lines, equal sized denticles; grey lines, equivocal denticles. In (c and d) black vertical lines on the right of the diagrams indicate hard substrate and grey shading indicates lacustrine taxa.

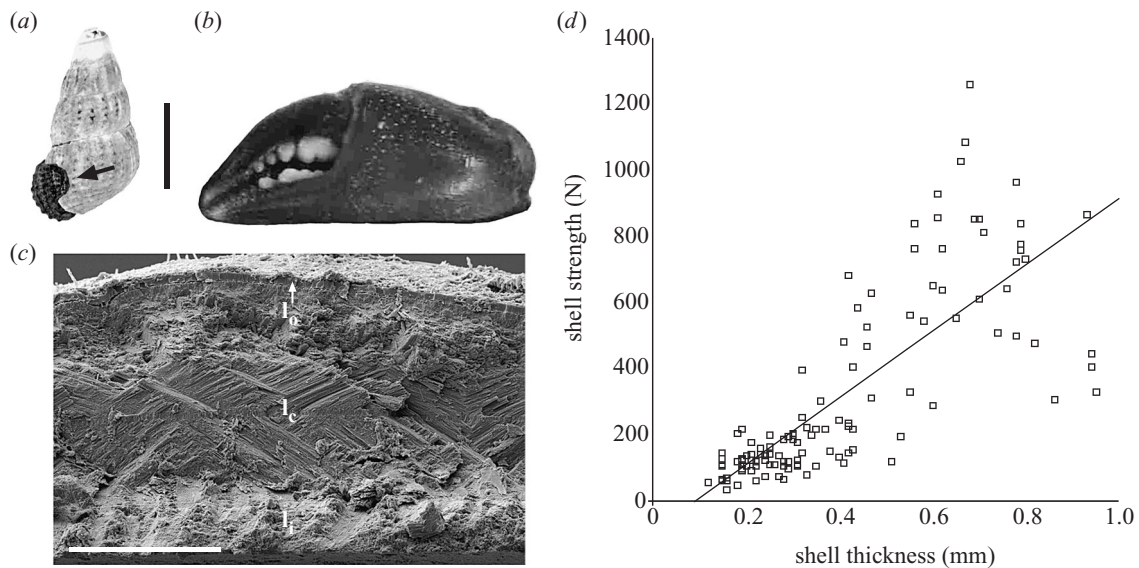


Figure 4. Shells and crab predation. (a) Shell with repair scar (*T. zeamais*, Lake Matano). Scale bar, 1 cm. (b) Left claw of molluscivorous lacustrine crab species (*Syntripsa matanensis*). (c) Shell structure of *T. perfecta* (riverine). l_o , outer layer; l_c , crosslamellar layer; l_i , inner layer. Scale bar, 0.1 mm. (d) Scatter plot showing correlation between shell thickness and shell strength.

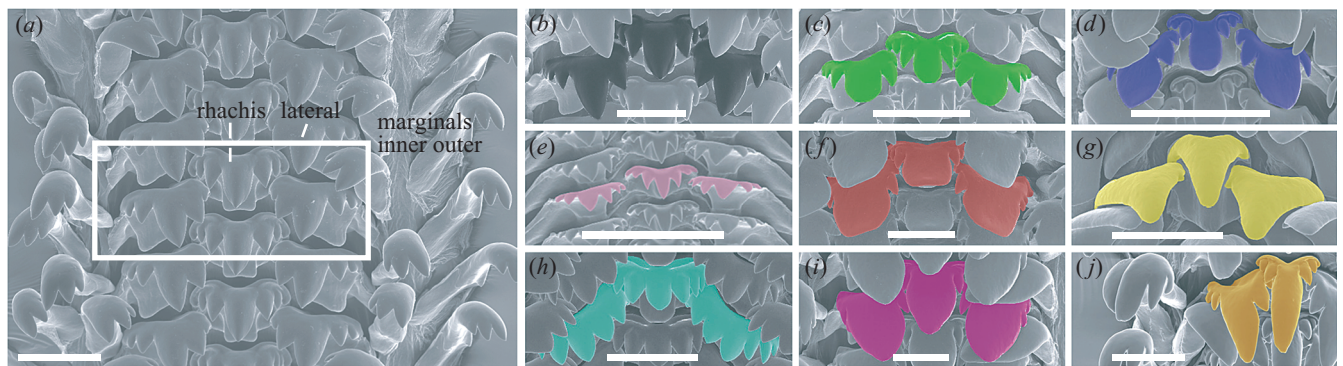


Figure 5. Radula types of *Tylomelania*. Colours correspond to those in figure 3d. Scale bars, 0.1 mm. (a) Segment, apical (45°), *T. perfecta*. The box indicates section shown in (b–k). (b) Typical pachychilid (riverine), *T. perfecta*; (c) short with dense dentation, *T. gemmifera*; (d) equal sized denticles, *T. kruimeli*; (e) enlarged major denticles, *T. towutica*; (f) large square major denticles, *T. sarasinorum*; (g) large round major denticles, *T. 'zeamais'*; (h) small spoon-shaped major denticles, *T. insulaesacrae*; (i) single hooked denticles, *T. carbo*; (k) long pointed major denticles, *T. spec Poso 2*.

The results presented here indicate the potential of the gastropod species flock in the ancient lakes of Sulawesi to serve as a further model system for the study of evolutionary factors in speciation and adaptive radiation. The indicated involvement of coevolution at a basal level in this system is unique among radiations in ancient lakes. In concordance with models developed for the evolution of fish species flocks (Sturmbauer 1998; Danley & Kocher 2001) which have recently been shown to be potentially applicable to vertebrate radiations in general (Streelman & Danley 2003), habitat and trophic specialization appear to be driving the initial stages of the Sulawesi radiation, though allopatric speciation may play a more prominent role in *Tylomelania*. Although species diversity in the Sulawesi lakes is low when compared with the cichlid radiations in East Africa, it is comparable to species numbers observed in other adaptive radiations, such as the

Galapagos finches or Caribbean *Anolis*. The gastropod species flock on Sulawesi appears to be lacking a third radiation phase fuelled by signalling (sexual) selection proposed by Streelman & Danley (2003), which may account for the amazingly high species diversity in cichlids. The applicability of models developed for vertebrate radiations to an invertebrate species flock with distinct intrinsic properties provides additional evidence that fundamental processes may govern adaptive radiations in insular habitats.

We believe that beyond the singular possibility to study escalation in the context of radiation, the relative simplicity of the Sulawesi system will especially favour tests of ecological speciation by comparing trait variance under different natural conditions. Recent work using highly variable nuclear markers (amplified fragment length polymorphisms) is expected to yield a highly resolved phylogeny at the

population and species level, and should allow, in conjunction with quantitative ecological data, an elaboration of our hypotheses on ecological speciation in the Sulawesi snails.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.