Speciation and Radiation in a River: Assessing the Morphological and Genetic Differentiation in a Species Flock of Viviparous Gastropods (Cerithioidea: Pachychilidae)

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Abstract The Kaek River in central Thailand is unique in harbouring a diverse species assemblage of viviparous gastropods of the genus *Brotia*. A stretch of this river less than 100 km long is inhabited by seven, mostly endemic species that are essentially differentiated by their shell morphology. Earlier, it has been suggested that this species flock fulfils some basic requirements of a radiation (monophyly and phenotype–habitat correlation). However, the present study has shown that there is no strict correlation between radula and shell morphology and the utilisation of sub-strates, such as rock or sand, thereby refuting the hypothesis that ecological speciation may have played a significant role. Phylogenetic analyses based on mtDNA show that haplotypes cluster together in drainage-specific clades rather than according to the taxonomy. There are also strong indications that introgressive hybridisation has occurred, which may have resulted from secondary contact of previously isolated species due to dispersal or river captures during the Cenozoic. It is assumed that the high species diversity in the Kaek River results from two phenomena that interdigi-tate. Firstly, the Kaek River fauna may have originated from multiple species invasions from different source areas, while traces of these events may have been obscured by introgression of Kaek River-specific haplotypes. Secondly, waterfalls in the Kaek River seem to affect the directionality and amount of gene flow between local populations within the river and several smaller tributaries. Together with temporally changing water regimes, this highly structured environment may have conserved local genetic differentiation and triggered diversification and speciation in peripheral isolates within relatively short periods of time.

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1   Introduction

Speciation in the context of (adaptive) radiations is regarded as a key process in creating biological diversity. Like oceanic islands, lakes have been found to provide ideal model systems for elucidating the underlying mechanisms of this evolutionary process. However, not only lacustrine but also riverine species flocks can potentially provide crucial insights into the study of speciation and adaptive radiation (see review of, e.g., Glaubrecht and Köhler 2004). Among invertebrates, limnic gastropods have been found to provide most suitable model organisms for these studies (see, e.g., Glaubrecht 1996, 1999, 2006). In addition to other freshwater gastropod radiations, such as in eastern African lakes (for review and recent literature, see Glaubrecht 2008) or the Indonesian islands of Sulawesi (Glaubrecht and Rintelen 2008; see also, in this volume, Rintelen et al. 2010), a unique and endemic species flock of closely related pachychilid gastropods is found in the Kaek River system in Central Thailand. Here, a total of ten species-level taxa (five species and five subspecies) were originally described from a restricted river stretch of less than 100 km in length, primarily with emphasis on the shell (Brandt 1968, 1974). Two of these species, *Brotia binodosa* and *B. paludiformis*, had been reported earlier by Solem (1966) from the Thung Salaeng waterfall. Subsequently, Brandt (1974) systematically revised the Thai species, but failed to recognise that he was presumably dealing with a radiation of closely related species. He affiliated the species from the Kaek River with either one of two distinct genera, *Brotia* and *Paracrostoma*. Although this treatment transpired the high levels of morphological distinctiveness in the shells of different species, at the same time it obscured the existence of a presumably monophyletic flock of morphologically well-differentiated species for decades. Davis (1982) first noticed the uniqueness of the Kaek River assemblage by stating that "when *Brotia* is found in rivers there is usually one species, two at the most. The exception to this is the small radiation in the Koek Noi River (=Kaek River) (north central Thailand) of the Nan-Chao Phraya drainage."

Our preliminary study of mitochondrial and morphological differentiation hinted at a potentially adaptive radiation in the riverine *Brotia* species from the Kaek River (Glaubrecht and Köhler 2004), very similar to the one found in the lacustrine *Tylomelania* on Sulawesi. In a first step, therefore, we revised the taxonomy of the Kaek River species based on examinations of types and newly collected, alcohol-preserved material. Confirming the existence of a remarkably diverse pachychilid fauna in the Kaek River, we recognised at least seven distinct and endemic *Brotia* species in the river (compared to the original ten species-group taxa). So far, in no other river has a comparable diversity of pachychilid species been found worldwide. Molecular analyses using mitochondrial sequences suggested monophyly of the Kaek River species flock but also revealed a rampant mismatch of the branching pattern of mtDNA-based phylogeny with the delimitation of species by their shell morphology. Morphological analyses and ecological observations suggested that the distribution of shell and radular morphs within the river may be correlated with the usage of certain substrates by the animals (i.e., soft

versus hard substrate dwellers exhibited divergent radular and shell morphologies). Based on these facts (monophyly, local endemism, mismatch between mitochondrial gene and species tree, correlation between phenotypes and environment), Glaubrecht and Köhler (2004) postulated that the Kaek River species flock originated through adaptive radiation possibly triggered by trophic specialisation along the evolutionary trajectories outlined for the confamiliar *Tylomelania* (Glaubrecht and Rintelen 2008; Rintelen et al. 2010).

In contrast to the lacustrine *Tylomelania*, which radiated in situ in the ancient lakes on Sulawesi, the Kaek River species flock has apparently evolved in a riverine environment. Riverine radiations are both rarely known and studied, with only a few known examples for gastropods, such as Asian Triculinae (Davis 1979, 1981), the Stenothyridae in the Mekong (Hoagland and Davis 1979), hydrobioid snails from Tasmania and Eastern Victoria (Ponder et al. 1993; Ponder et al. 1994), and bithyniid snails in West Africa (Brown 1988). In all these cases, however, the exact causes of radiation remained hypothetical.

Therefore, it was the aim of the present study to compare cases of intralacustrine and intrariverine radiations in this group of closely related pachychilid gastropods in order to improve our understanding of the relevance of environmental factors for the evolution of invertebrate species flocks. It has been our goal to unravel the origins of the Kaek River species flock and to reconstruct the spatial and temporal patterns of its evolution, using a combination of molecular and morphological studies. We were also interested in identifying those factors that have been driving the morphological and genetic differentiation of these species. The patterns of morphological and genetic differentiation within and among the Kaek River species were studied with emphasis on possible correlations between morphological traits and environmental factors. Recently, the ecological component of speciation received much attention, with habitat selection, trophic specialisation and sexual selection being identified as key factors promoting speciation in sympatry and, potentially, also adaptive radiation (e.g. Schluter 2000; Streelman and Danley 2003; Gavrilets and Losos 2009). In order to assess whether we are dealing with a truly adaptive radiation driven by ecological speciation in the case of the Kaek River pachychilids, we addressed four of the main criteria, viz. monophyly, rapid speciation, phenotype–environment correlation, and trait utility as suggested by Schluter (2000).

2 The Systematic Framework: Phylogeny of the SE Asian Pachychilidae

Pachychilidae Troschel, 1857 is a group of freshwater gastropods only recently recognised as an independent freshwater radiation within the diverse and otherwise predominantly marine gastropod superfamily Cerithioidea Férussac, 1819 (Glaubrecht 1996; Lydeard et al. 2002; Köhler et al. 2004). Novel studies of

pachychilids provided insights into speciation in the context of adaptive radiation (Glaubrecht and Köhler 2004; Rintelen et al. 2004, 2007; Glaubrecht and Rintelen 2008) as well as evolutionary phenomena, such as the development of parental care in these viviparous snails (Köhler et al. 2004). Within the Pachychilidae, oviparity is considered a plesiomorphic trait (Glaubrecht 1996, 1999, 2006; Köhler et al. 2004), and is found in the African (*Potadoma* Swainson, 1840), Malagasy ("*Melanatria* Bowdich, 1822" [name replaced by *Madagasikara* Köhler and Glaubrecht, 2010]), and Neotropical (*Pachychilus* I. and H.C. Lea, 1850, *Doryssa* Swainson, 1840) taxa (Binder 1959; Grossmann 1967; Starmühlner 1969; Brown 1994; Simone 2001). By contrast, in Southeast Asia – where this gastropod family is particularly diverse – pachychilids are (ovo)viviparous throughout (Brandt 1974; Köhler and Glaubrecht 2001, 2005, 2006, 2007; Glaubrecht and Rintelen 2003; Rintelen and Glaubrecht 2003, 2005; Köhler et al. 2004; ; Rintelen et al. 2007) (Fig. 1). However, in conflict with more traditional assumptions (e.g. Morrison 1954; Brandt 1968, 1974; Glaubrecht 1996), the brooding taxa in South and Southeast Asia do not form a monophyletic group. Instead, three distinct clades have been identified by analyses of morphological and molecular data (Köhler and Glaubrecht 2001; Köhler et al. 2004; Köhler and Dames 2010). In terms of their morphology, these clades are mostly characterised by their reproductive anatomy: Within the genus *Jagora* Köhler and Glaubrecht 2003 (clade 1), females retain yolk-rich eggs in the mantle cavity from which the hatchlings are released. Yolk delivered with the egg capsule represents the only form of nourishment provided by the mother (Köhler and Glaubrecht 2003). In contrast, *Tylomelania* F. & P. Sarasin, 1898 and *Pseudopotamis* Martens, 1894 (clade 2) are eu-viviparous and possess a brood pouch formed from the pallial oviduct. The retained embryos are nourished for a considerable period of time by maternal albumin secreted by the mother (Glaubrecht and Rintelen 2003; Rintelen et al. 2007). Finally, representatives of the third clade possess a subhaemocoelic brood pouch. In these species, nutrients are provided exclusively by the egg capsule; any kind of secretory tissue is absent from the incubatory pouch (Köhler and Glaubrecht 2001).

Fig. 1 Backbone tree showing the relationships of pachychilid genera as inferred from analyses of partial 16S sequences by Köhler et al. (2004). Brooding taxa are *shaded*, oviparous taxa are not shaded. The most parsimonious explanation is that the three brooding strategies pursued by different pachychilid clades have evolved independently while oviparity represents a plesiomorphic state within the family (note that *Melanatria* has recently been replaced by *Madagasikara*)
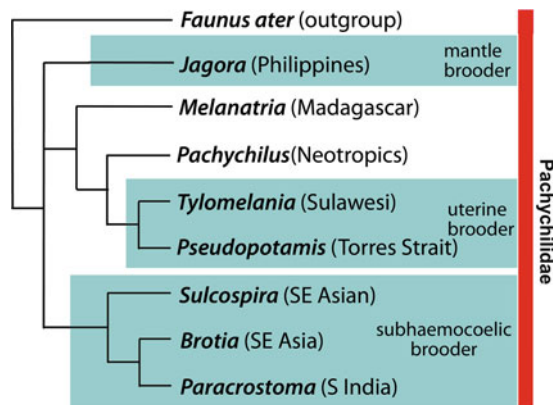
The three Asian clades of brooders are spatially well separated: *Jagora* is endemic to the Philippines, *Tylomelania* and *Pseudopotamis* are restricted to Sulawesi and two Torres Strait islands (northern Australia), respectively, and the subhaemocoelic brooders display an extended Sundaland distribution occurring from India to southern China, including the Malay Peninsula, Sumatra, Java, and Borneo. The latter clade has been referred to as the "Asia mainland clade" (Köhler et al. 2004; Köhler and Glaubrecht 2007; Köhler and Dames 2009). Compared to the two other Asian clades, the subhaemocoelic brooders display a much wider distribution, larger variation with respect to their morphology and in total a higher diversity of species. Various, in part conflicting, generic classifications were suggested for members of this heterogeneous group. Between two and four genera were delimited (*Sulcospira* Troschel, 1858, *Brotia* H. Adams, 1866, *Paracrostoma* Cossmann, 1900, *Adamietta* Brandt, 1974) by various twentieth century authors, such as Thiele (1928, 1929), Morrison (1954), Benthem Jutting (1956), and Brandt (1974). In general, these genera were established on the basis of certain shell, opercula and/or radular features – characters that subsequently proved not to be appropriate at this taxonomic level due to homoplasy (Köhler and Glaubrecht 2001, 2002, 2005, 2006, 2007). For instance, recent studies have shown that shell shape and sculpture often reflect ecological adaptation rather than phylogenetic relationships, with cases of remarkable parallelism being discovered in these pachychilids (Köhler et al. 2008).

Based on a comprehensive taxon sampling that covers the entire range of the group from southern India in the west to southern China in the east and Borneo in the south-east, Köhler and Dames (2009) have addressed the question of monophyly of the nominal genera of mainland Pachychilidae and analysed partial sequences of the mitochondrial genes COI and 16S as well as key morphological characters, notably the female genital anatomy and embryonic shell morphology. They suggested recognition of three genera (*Brotia*, *Paracrostoma*, *Sulcospira*) among the SE Asian subhaemocoelic brooders of mainland SE Asia (Fig. 2). Within *Sulcospira*, which represents the most basal offshoot of the clade of subhaemocoelic brooders, three sub-clades have been recognised that reveal a largely geographical structuring (with lineages each in Borneo–SE Asia mainland, Southern China–Vietnam, and Java–Borneo). All *Sulcospira* species exhibit widely congruent gross morphologies, however, this being the reason to refrain from formally naming these clades. In addition, the molecular phylogeny of the SE Asian mainland Pachychilidae provided evidence for the existence of a monophyletic clade of *Brotia* species in Central Thailand, which contains species that are endemic to the river systems of the Kaek and Kwae Noi River (Nan drainage), the Loei and Pong River (Mekong drainage), and the Pa Sak River (Chao Praya drainage).

Previous analyses of the rates of mitochondrial and morphological differentiation among the Asian Pachychilidae revealed two independent species flocks, which are characterised by (1) monophyly, (2) close relationships between their constituent members, (3) considerable degrees of interspecific morphological differentiation with respect to shell and radula, and (4) rampant mismatch of mtDNA phylogenies and morphology-based species delimitations. Both species flocks,
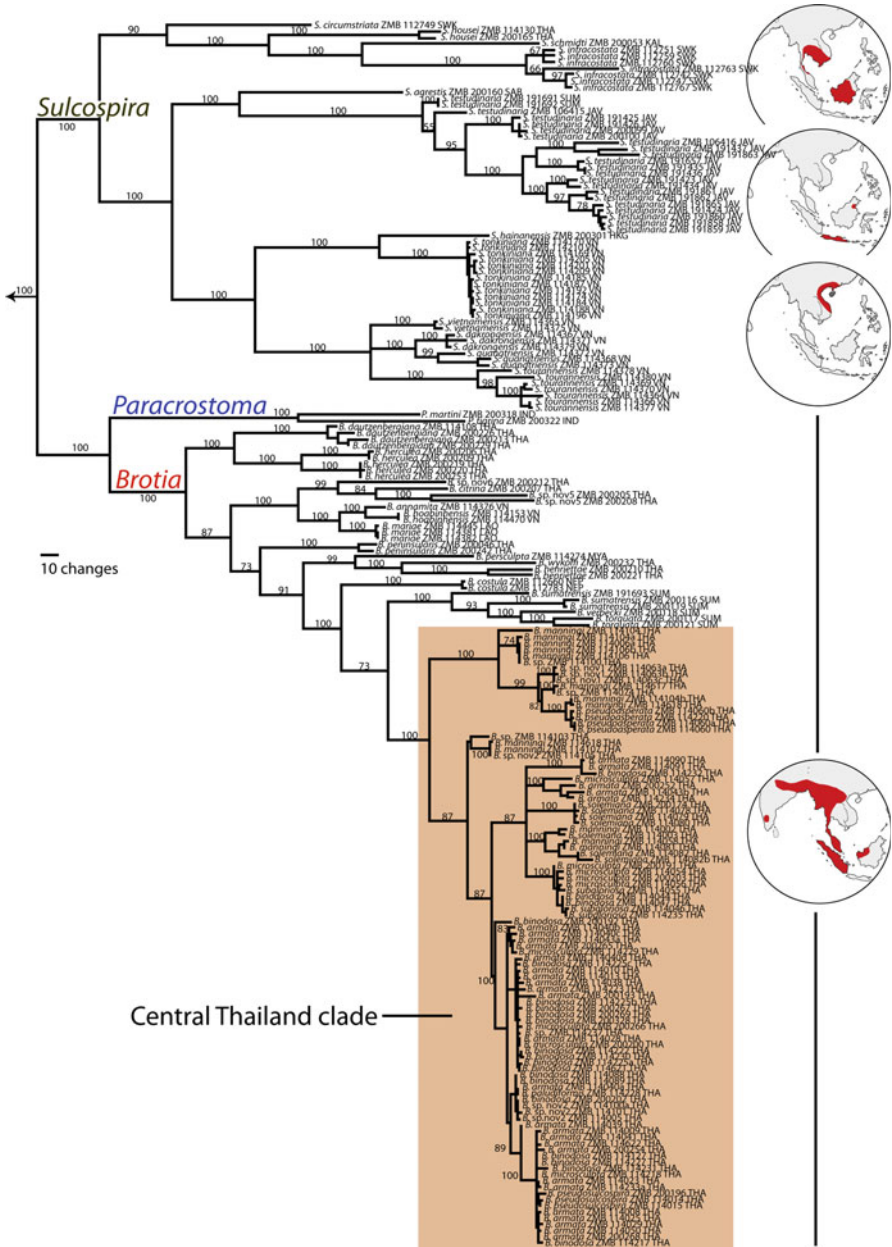
Fig. 2 Phylogenetic relationships within the clade SE Asian mainland clade of subhaemocoelic brooders (genera *Brotia*, *Paracrostoma*, *Sulcospira*) as inferred by analyses of concatenated COI and 16S sequences (Köhler and Dames 2009). The Central Thailand clade of *Brotia* is *shaded Area codes*: HGK, Hong Kong; IND, India; JAV, Java; KAL, Kalimantan; LAO, Laos; MYA, Myanmar; NEP, Nepal; SAB, Sabah; SUM, Sumatra; SWK, Sarawak; THA, Thailand; VN, Vietnam

Tylomelania in the Central Lakes of Sulawesi and *Brotia* in the Kaek River, Central Thailand, have been postulated to have resulted from adaptive radiations. The model case of *Tylomelania* on Sulawesi has been extensively studied for a period of almost 10 years, and ongoing work has shown that these endemic freshwater gastropods have radiated extensively in the two ancient lake systems of the island (Rintelen and Glaubrecht 1999, 2005; Rintelen et al. 2004, 2007; Glaubrecht and Rintelen 2008; see also, in this volume, Rintelen et al. 2010).

3   The Kaek River: Geographical and Environmental Settings

Knowledge of the geological history and the current environmental conditions in the Kaek River drainage is relevant for the understanding of the origin of the species flock and the significance of abiotic factors that may have influenced its evolution. The geological and hydrological data presented here has been gathered from various sources, such as topographical maps and online facilities. Note that due to the absence of a generally binding transliteration from Thai to English, locality names as spelt herein may differ from versions used elsewhere. With respect to localities within the Kaek River area, we preferentially refer to names as firstly spelt by Brandt (1968, 1974) for the sake of continuity while otherwise we refer to spellings as used in the current edition of the Times Atlas of the World.

The Kaek River (Maenam Kaek in Thai, also called Klong Talo at its lower reaches; Brandt 1968) flows into the Nan River near the city of Phitsanulok. The Maenam Nan is a first-order tributary of the Chao Praya, which is a broad, moderately fast-flowing river that winds its way through the central plain of Thailand and discharges into the Bay of Bangkok. The Chao Praya basin can be divided into two parts. The lower part is flat at low altitudes and extends towards the north as far as Ang Thong (ca. 15°N). This basin is filled with Quaternary deposits and was flooded for the last time by the South China Sea about 9,000–10,000 years ago when sea levels were ∼4 m higher than today. The upper plain extends northwards up to the valleys of the Nan and Ping Rivers. This plain lies at elevations of more than 20 m above sea level and has not been subject to significant tidal flooding in the more recent past. The upper reaches of the watershed are located at ∼19°N, in the provinces of Mae Hong Son, Chiang Rai and Chiang Mai.

The Kaek River flows in an E–W direction from the watershed west of Phetchabun towards Phitsanulok. It is located within the transition area between the Nan-Uttaradit suture zone, which is demarked by the Nan River valley between Nan and Saraburi, and the Loei-Phetchabun foldbelt (Cooper et al. 1989). Being situated at higher elevations within the ranges that are part of the Thung Salaeng Luang National Park, the upper and middle part of the river are located at the western fringes of the Loei-Phetachbun foldbelt whereas its lower course

(Klong Talo) between Wang Thong and Phitsanulok reaches the lower and flatter areas within the Nan valley (Chonglakmani and Helmcke 2001). The upper to middle course of the Kaek River has cut a steep-sloped canyon into an area formed essentially by Permian limestone as well as Jurassic sandstones, slate and hardpan across the Thung Salaeng Luang ranges (DNP 2009) between altitudes of 300 and 1,028 m.

For most of its ∼150-km-long course east of Wang Tong, the Kaek River is a fast running stream. Its water is clear and relatively cold. The upstream region is characterised by a moderate decline and grounds of gravel and stones. Midstream waters flow swiftly over a rocky bottom with large boulders where they pass a series of rapids and waterfalls on their way west. Between the rocky sections, there are also sections with a more moderate decline in which a reduced flow regime results in the deposition of large amounts of sand and mud that form the main substrate here. But in general, soft substrates are rare in the upper and middle course and may provide only unstable conditions depending on the seasonally variable water regime. On the other hand, in the lower course between Wang Tong and Phitsanulok, only sandy to muddy substrates are found. Pine and bamboo forest as well as mixed species deciduous forest dominate the area surrounding the river, while grassland, lowland scrub and tropical broad-leaved evergreen forest cover smaller areas. Human impact is rather limited (mostly in bathing areas, near settlements), but increases in the downstream region towards Phitsanulok with its expanded farmland. Although the Kaek River is continuously supplied with water, the amount of water changes seasonally. In and shortly after the rainy season from around June through October, significantly more water flows down the river than in the dry season between November and April. During the rainy season, the Kaek is a wild-water stream, while during the dry season, the current is moderate and some of the smaller affluents and headwaters even become entirely dry. Limnological data on rivers and streams in tropical Asia are scarce (Dudgeon 1995). As predicted by the river continuum concept (Vannote et al. 1980), streams and their organismic composition and diversity are characterised by a flowing continuum, with distinct reaches not being delimited by fixed borders. However, in terms of the broadly used geomorphic or physiographical stream classification (Allan 1995; Hauer and Lamberti 1996; Giller and Malmqvist 1998), we interpret the Kaek River herein to represent a medium to large river of third order (with the Chao Praya and Nan River being mainstream rivers). According to the more useful biotic river classification scheme developed by Illies (1961), we classify the Kaek River herein to be a rhithral or middle stream section with its organismic components representing the rhitron. The rhitral is typically characterised by rather cool temperatures, high to moderate dissolved oxygen concentrations (often variable at least seasonally), with water ranging from clear to turbid and oligotrophic to mesotrophic, rather variable medium (semistable) substrates and stronger currents with a comparatively high gradient. The Kaek belongs to the Chao Praya biogeographical region established for freshwater fishes (Yap 2002); for details of zoogeography see Rainboth (1996).

4 River Capture: Paleogeography and Palaeohydrology

In the Cenozoic, SE Asian rivers were affected by two major geological processes: sea level fluctuations and realignments caused by tectonic changes or erosion. Sea level fluctuations have constantly changed coastlines due to the flooding or surfacing of vast areas. For instance, sea levels were apparently higher than today during the Miocene (+150–220 m, at 24–13 mya) and Pliocene (+100 m, at 5.5–4.5 mya) (Woodruff 2003), while they have been considerably lower during the Pleistocene (up to 120 m below today's level; Martinson et al. 1987). Sea levels of +100 m or more would have resulted in a northward extension of the Gulf of Siam and flooding of large parts of the Chao Praya river basin. However, even then, the Kaek River in its current configuration would not have been submerged as it lies at even higher altitudes. It may have, however, lost its connection to other parts of the Chao Praya drainage system.

Changes in drainage configuration of rivers in Central Thailand may have been more relevant in this regard. Gregory (1925) first pointed out that, during the Cenozoic, the major river systems of Central and Southeast Asia underwent dramatic changes due to tectonic processes, such as the uplift of areas and lava flows. The history of these river systems has been described in more detail by Hutchinson (1989) and Rainboth (1996). According to these reconstructions, the Chao Praya lost its headwaters to the growing Mekong in the middle and upper Pleistocene. Until around 2 mya, the Irawaddy, Salween and Mekong drained into the Chao Praya, unless around 1.5 mya volcanic activity separated the Irrawaddy and Salween rivers from this system. Since then, the Mekong changed its river bed repeatedly to successively more easterly directions. After its midstream has been separated from the Salween around 2 mya, its course followed the present course of the Ping River (Chao Praya drainage) until around 1.5 mya. Late Cenozoic faulting diverted the Mekong further eastwards along its present course towards Vientiane until, later in the mid-Pleistocene (∼1 mya), the Mekong once again drained into the Chao Praya, this time via the valley of the Loei and Pa Sak Rivers. Eventually, it changed its course again around 50,000 years ago towards the east, where it has undergone further course changes.

While the details and exact timing of the geological history of the Mekong drainage are not fully understood (Gupta 2008), it is clear that the courses of smaller rivers were also affected by tectonic processes. Some of them even reversed their original direction of flow due to uplifts that affected their upper or mid-streams, such as the Loei River that was once part of the southward-flowing proto-Mekong but today flows in a northward direction, or the Mun River that once drained in a westerly direction into the Chao Praya until it reversed its course towards the east due to the sinking of the Khorat Plateau during the mid-Pleistocene (Hutchinson 1989). On a smaller scale, the details of the geological history of the Kaek River area are difficult to reconstruct. The headwaters of the westward-flowing Kaek River, the southward-flowing Pa Sak River, and the northward-flowing Loei River are in close conjunction, separated by the up to 1,700-m-high mountain ridges of

the Phang Hoei Range, which are of relatively recent (Cenozoic) igneous origin. Prior to the uplift of these mountains approximately during the Pliocene, there was likely a single river flowing in a N–S direction through the beds of the Loei and Pa Sak River (Hutchinson 1989). It can only be speculated as to how the river systems looked like before this period and if the upstream region of the Kaek River was also connected to the proto-Mekong drainage at this time. Later, from the mid-Pleistocene until 0.05 mya, the Mekong flowed through the beds of the present Loei and Pa Sak rivers again and re-connected their once separated faunas. The Kaek River itself has likely not been affected by these more recent reconfigurations of drainage systems as its upstream region was probably already at higher elevations. However, it is clear that hydrological phenomena such as river captures have been effective in the whole area with respect to the connection and separation of drainage systems, which must have also influenced their biota as suggested by Glaubrecht and Köhler (2004).

5   Sampling Design and Collection Sites

In order to account for the possible relevance of ecological factors, we generally collected specimens that occurred on different substrates (rock, wood, sand, mud) separately, and also differentiated between specimens collected at different depths (at levels of 0, 0.5, 1, 1.5 and 2 m water depth). To address the relationships on a larger scale and to reveal the origins of the Kaek River species flock, we collected *Brotia* samples in all adjacent river systems of the Kaek River, viz. the drainages of the Kwae Noi in the north, the Loei in the northeast, the Pa Sak in the southeast and the Pong in the east. The drainages of the Kwae Noi and the Kaek are separated from each other by the southern extensions of the Luang Prabang Range with mountainous ridges reaching elevations of 1,035 m, 1,356 m (at and near the Khao Kho), 1,746 m (Phu Hin Ronkla), and 1,468 m (Phu Khat) (from S to N). Towards the northeast, these ranges separate the catchment areas of these two rivers from the adjacent drainage of the Loei River, which flows northward into the Mekong. The headwaters of the Pong River, which flows to the east via the Mun River into the Lower Mekong, are located in the east of the Phang Hoei Range while the Pa Sak River flows towards the south and discharges into the Chao Praya (Fig. 3).

   This material basis was complemented by collections from other parts of Thailand and neighbouring regions of Laos in an attempt to cover all major drainage systems of the region, i.e. the Salween (with its first order tributary Maenam Moei) in western Thailand, the Chao Praya (with its two principal tributaries Nan and Ping) in central Thailand, and the Mekong (with its tributary Mun) in north-western Thailand and Laos (Fig. 4).

   The most extensive sampling was undertaken within the Kaek River drainage, however. Not all sectors of the Kaek River were accessible during this study either due to the rugged topology or due to access restrictions in the Thung Salaeng
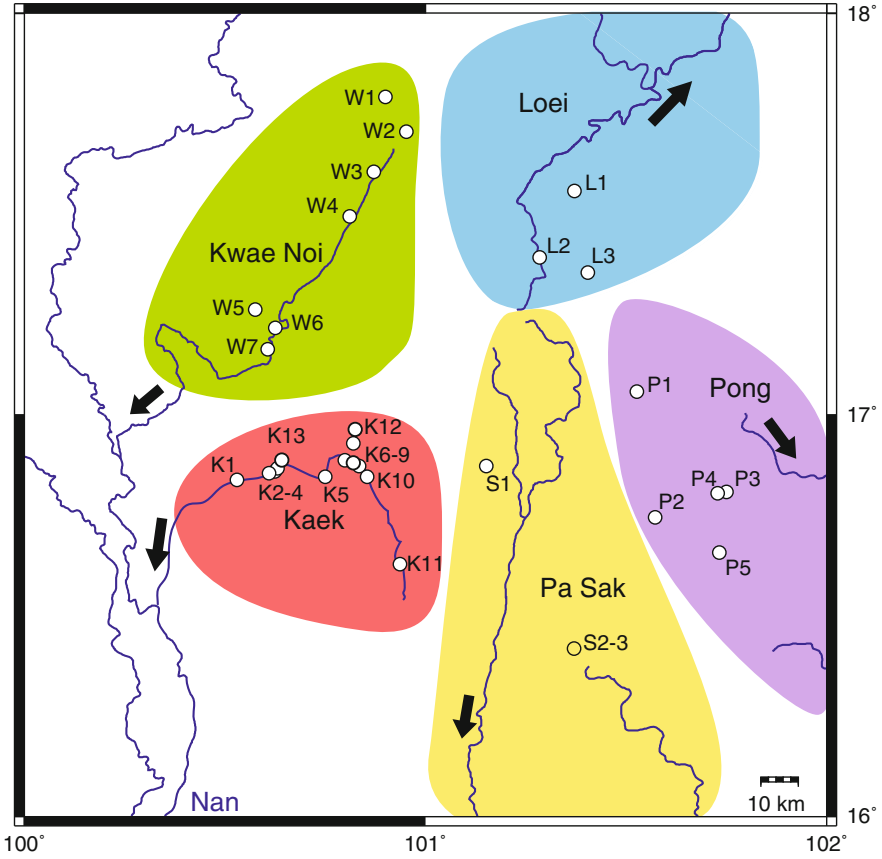
Fig. 3 Collection sites in north-central Thailand and their location within the catchment areas of the five main rivers that drain the Phetchabun Mountains towards the west (Kaek and Kwae Noi River, tributaries of the Nan River), north (Loei River, tributary of the Mekong), east (Pong, tributary of the Mun), and south (Pa Sak River, tributary of the Chao Praya)

Fig. 4 Topographical map showing the location of collection sites in Thailand and Laos, field work in 2006–07. *Frame in centre* delimits the area depicted in Fig. 05. *Red dots* mark sites where *Brotia* species were found, *white dots* where no *Brotia* species were found. Accordingly, *Brotia* is confined to mountainous regions of NW and W Thailand but absent from the plains in central, south, and east Thailand
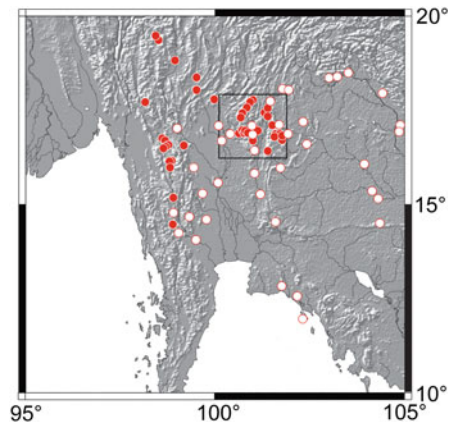
National Park or on private properties. Between Wang Tong and the Headquarters of the Thung Salaeng National Park at the Thung Salaeng rapids, the National Road 12 from Phitsanulok to Lom Sak runs parallel to the midstream segment of the river. Alongside this road, there are several areas that are within easy reach, mostly near or at waterfalls and rapids that are signposted as tourist attractions and also used for recreational purposes by local tourists. The material first described by Brandt (1968, 1974) mostly originates from these sites. In his descriptions, Brandt (1974) referred to the road distances of the sampling sites along the highway 12 from Phitsanulok. We continue to refer to these distances to ensure comparability of ours and his data; a reference number is assigned to each of them. Since the Kaek River flows in a westward direction towards Phitsanulok, in the following the sampling sites are listed in an upstream order (Fig. 5). In addition to the sampling sites referred to by Brandt (1968, 1974), our work covers further sites along the river course as well as in permanently water-filled affluents of the Kaek River; some of which were found not to harbour *Brotia* species. Between the Nan River near Phitsanulok and Wang Tong, the Kaek River flows through a plain on muddy to sandy substrates. No *Brotia* species were found in this lower segment. East of Wang Thong, the area ascends steeply to higher elevations, which marks the end of the fast-running midstream region. The first accessible sampling site are the Sakunothayan rapids at km 33 (K1). This spot is followed by a smaller affluent that flows over rocks (km 37), where we collected samples at around 8 km distance from the main stream of the Kaek River (K2). The third spot is an area with unnamed rocky rapids at km 42 of the highway (K3), followed by the Kaeng Song waterfall
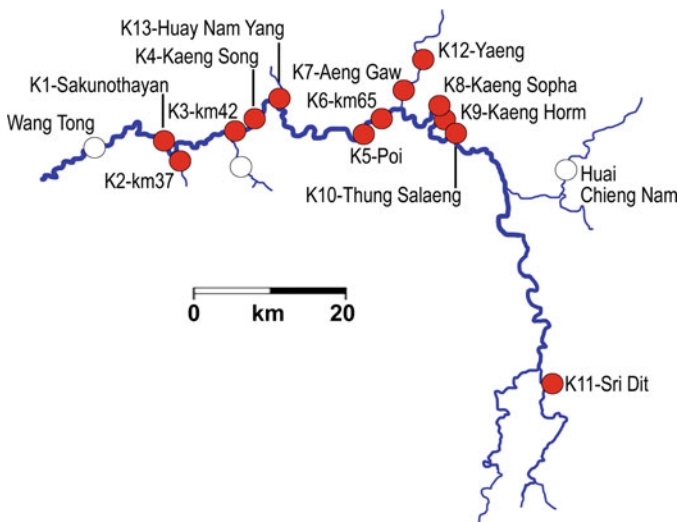

Fig. 5 Location of collecting sites at the Kaek River, field work in 2006 and 2007. *Red dots* mark localities at which *Brotia* samples were found, *white dots* mark localities at which no *Brotia* species was found

at km 45 (K4), a small affluent from the north with rocky substrates, the Huay Nam Yang (K13), the Poi waterfall at km 60 (K5), a further area with unnamed rocky rapids at km 65 (K6), the Aeng Gaw waterfall, situated in an small affluent from the north at km 67 (K7) that flows through the village of Yang (K12), the Kaeng Sopha waterfalls at km 72 (K8), and the Kaeng Horm rapids at km 73 (K9). The Thung Salaeng rapids at km 76 (K10) are the last collection site along the National Road 12. From here, the Kaek River flows through protected and inaccessible areas of the Thung Salaeng National Park. The next and last sampling site is the Sri Dit waterfalls (K11) about 40 km SE of Thung Salaeng. Sites that were found not to harbour *Brotia* species are the lower portion of the Kaek River near Wang Tong, an affluent from the south near km 42, and the affluent Huay Chieng Nam east of Thung Salaeng. The latter creek is the type locality of *Brotia subgloriosa* (Brandt 1974). However, complete deforestation and degradation of the whole area have obviously affected this river, which is now a slow-flowing, muddy creek that is not suitable for *Brotia*.

The configuration of the collection sites differs to a certain degree (Fig. 6). Rocks are the predominant substrate across the entire length of the Kaek River. At waterfalls within the main course of the river (at Kaeng Song, Poi, Kaeng Sopha), the water runs over large steps with heights from 1 to 4 m. At rapids, the water runs swiftly over a broader stretch of rocks and boulders. There, water depth is usually low (less than 0.5 m) and sandy patches are absent. In between the waterfalls and rapids, there are also quieter areas with moderate currents and depths of up to 2.5 m. Here, sandy and muddy substrates are found to cover the rocky bottom of the river bed. Sandy patches were found near Sakunothayan in depths of 0–2 m and muddy patches at Kaeng Song and near km 45 at depths of around 2 m. Smaller areas with sand between larger rock fields were also found at Thung Salaeng and Kaeng Sopha in depths of around 0.5–1 m.

6 Patterns of Shell Variation Among and Within the Kaek River Species

Compared to most regions in SE Asia, the Kaek River harbours an exceptionally diverse pachychilid fauna with respect to the species composition as well as the variability found in the shells of these species. Brandt (1968, 1974) initially delimited ten species-group taxa, of which seven were subsequently also recognised by Glaubrecht and Köhler (2004), all of them being considered as distinct species, essentially discriminated by means of their shell shape and sculpture. Accordingly, among the Kaek River species shells vary from elongate and sculptured (*B. binodosa*) via conical and sculptured (*B. armata*), to globular and smooth (*B. paludiformis*), broadly conical and smooth (*B. pseudosulcospira*), elongately conical and smooth (*B. microsculpta*) or elongately turreted and smooth (*B. subgloriosa*) (Fig. 7).
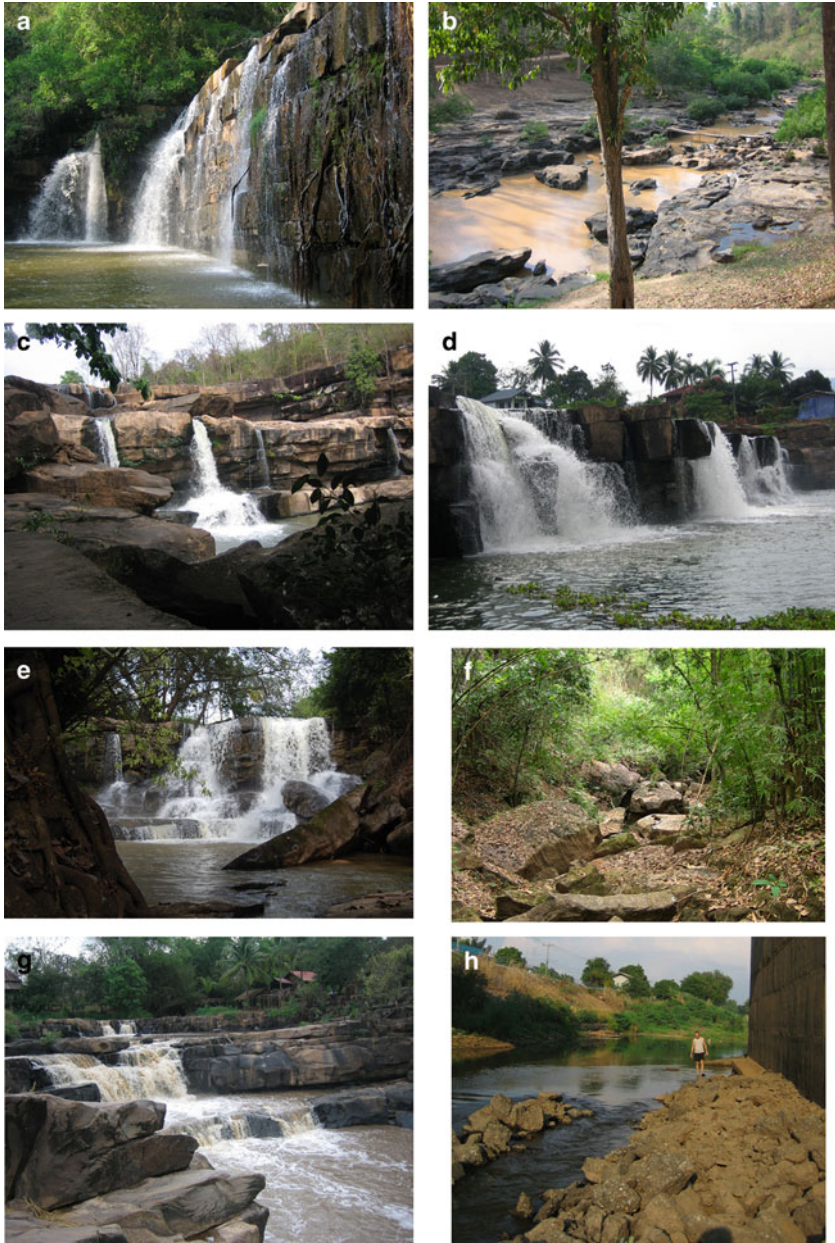
Fig. 6 Collection sites at the Kaek River (in downstream order). (**a**) Sri Dit waterfall (K11).
(**b**) Thung Salaeng rapids (K10). (**c**) Kaeng Sopha waterfall (K8). (**d**) Poi waterfall (K5). (**e**) Aeng
Gaw waterfall (K7), end of rainy season, November 2007. (**f**) Aeng Gaw waterfall, end of dry
season (K7). (**g**) Kaeng Song waterfall (K4). (**h**) Lower course of Kaek River in Wang Tong. All
photos except of (**e**) were taken in February 2006 at the end of the dry season. Specimens were
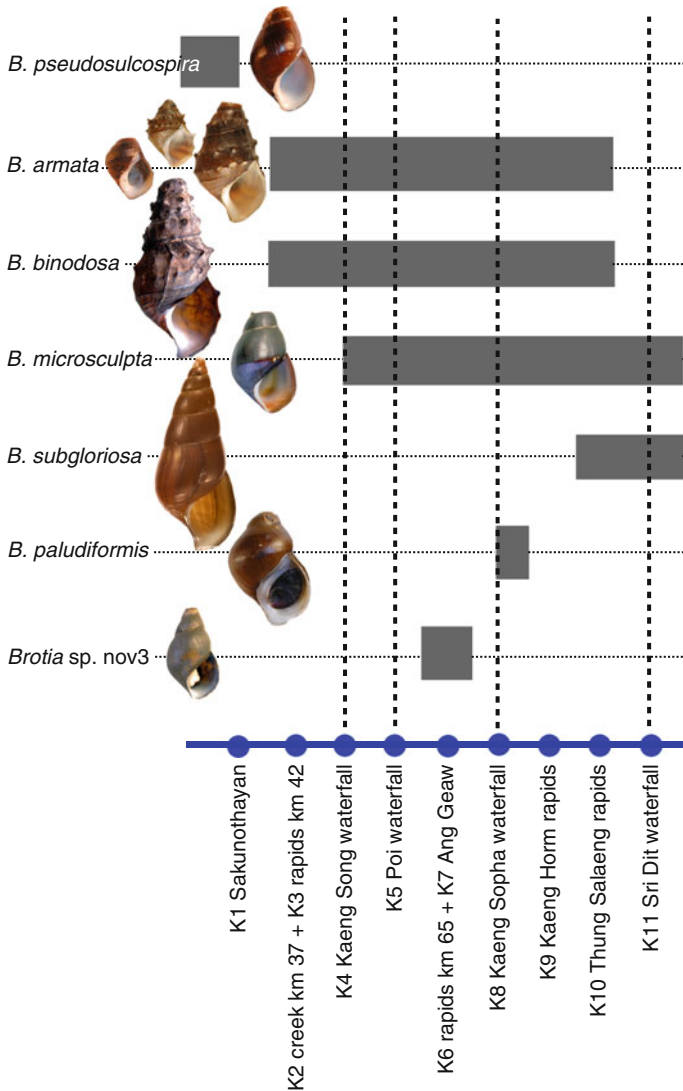collected above and below the waterfalls

Fig. 7 Distribution of *Brotia* species within the Kaek River. *Vertical lines* indicate barriers in the river course formed by waterfalls. Note that the Aeng Gaw waterfall is not situated directly in the main stream of the Kaek River but part of an affluent creek

The results of the current study are based on the most comprehensive basis of material, which also includes newly collected samples from various localities within and outside the Kaek River drainage that were not available to previous workers. They widely confirmed the taxonomical treatment of the *Brotia* species in the Kaek River by the latest systematic revisions (Glaubrecht and Köhler 2004;

Köhler and Glaubrecht 2006). Only a few details are considered to be in need of revision, as will be outlined in the following. However, we here refrain from a formal taxonomic treatment and instead refer to informal names where considered necessary. Only short diagnoses for species are presented here for the sake of readability of the text; for more comprehensive descriptions, we refer to previous taxonomic treatments, such as Brandt (1968, 1974), Glaubrecht and Köhler (2004), and Köhler and Glaubrecht (2006). See Table 1 for a general comparison of shell parameters.

Brotia pseudosulcospira has an almost limpet-like shell with no more than two whorls. It is generally smooth and thick-shelled and has a wide and ovate aperture as well as a large, oval operculum that almost fits the aperture. The body whorl comprises most of the shell height. It is well rounded in diameter; a slight depression below the upper suture being visible. This species only occurs at the Sakunothayan rapids (K1), and no other congener has been found to co-occur. *Brotia armata* was reported in error from Sakunothayan by Glaubrecht and Köhler (2004).

Brotia armata is widespread, being found in the Kaek River between the Kaeng Song rapids (K3) and the Thung Salaeng rapids (K10), in two creeks that discharge into the river (at km 37 (K2) and Huay Nam Yang (K13)), as well as in the drainage of the Kwae Noi River (W6, W3). It is not only the most widespread but also the most variable species with respect to its shell. Shells are typically sculptured with two to four spiral ridges, of which one or two may support spiral rows of pointed nodules or small spines at the periphery of the whorl. However, some shells are almost entirely smooth. Shells comprise between two and three whorls; the body whorl being inflated and proportionally considerably larger than the preceding whorls. The operculum is oval and almost fits the aperture. Apart from this general pattern, local populations differ considerably in shell shape and sculpture: almost limpet-like specimens with only a single whorl were found at lower reaches of the Kaek River (Kaeng Song, K3), while specimens with up to three whorls were found in upper midstream regions of the Kaek River (between Poi and Thung Salaeng) as well as in the Kwae Noi (W3). Populations within the Kaek River showed mostly a rather weakly developed sculpture whereas specimens collected in an affluent creek at km 37 (K2) exhibited a well-developed sculpture including the presence of spines. For spiny specimens like these, Brandt (1974) introduced the subspecies name '*morissoni*', which has subsequently been considered a synonym of *B. armata* by Glaubrecht and Köhler (2004). This treatment is still considered correct, since we found all transitions from spiny to the complete lack of spines within this population, which is considered as evidence that the occurrence of phenotypes that differ with respect to presence and development of spines is controlled by environmental factors, such as, possibly, water current and predation. *Brotia armata* differs from *B. pseudosulcospira* by its relatively more inflated body whorl, the absence of a sub-sutural depression, and the presence of usually well-developed sculptural elements.

Brotia binodosa occurs in the Kaek River between Kaeng Song and Thung Salaeng, in a tributary at Yaeng (K13), and in the Kwae Noi. Three shell forms can be differentiated, (1) a large, thick-shelled, and broadly conical form with

Table 1 Shell parameters (mm) of Kaek River *Brotia* species (means and standard deviation)

	B. pseudosulcospira	B. armata	B. binodosa (A)	B. binodosa (B + C)	B. microsculpta	B. paludiformis	B. subgloriosa	B. sp. nov3
Shell height	27.1 ± 2.5	18.1 ± 4.8	34.5 ± 4.9	27.7 ± 6.8	15.1 ± 3.1	23.2 ± 3.9	37.7 ± 3.0	14.8 ± 3.6
Shell breadth	17.5 ± 1.5	13.3 ± 3.4	22.9 ± 1.9	15.7 ± 3.4	9.8 ± 1.7	18.2 ± 2.6	19.3 ± 1.6	8.6 ± 2.1
Aperture length	17.4 ± 1.8	12.2±2.9	20.3 ± 1.7	14.0 ± 2.9	9.0 ± 2.2	16.0 ± 2.4	15.0 ± 0.9	7.7 ± 1.9
Aperture width	10.8 ± 1.3	7.9 ± 2.0	12.0 ± 1.1	8.5 ± 2.0	5.5 ± 1.2	11.3 ± 2.0	10.1 ± 0.8	4.8 ± 1.3
Body whorl	24.0 ± 2.2	16.3 ± 4.5	28.4 ± 2.5	20.6 ± 4.7	12.4 ± 2.1	21.2 ± 3.5	24.4 ± 1.5	11.5 ± 2.8
Whorls	2.1 ± 0.4	2.0 ± 0.8	2.5 ± 0.7	3.2 ± 0.9	2.6 ± 0.7	1.8 ± 0.3	4.2 ± 0.6	3.2 ± 0.9

pronounced spiral ridges that support up to two spiral rows of rounded nodules, (2) a small form with conical shells and pointed tips with rather flattened whorls and one or two spiral rows of well-developed spines, and (3) a form with more elongated shells that usually exhibit a sub-sutural depression and one or two spiral rows of weakly developed spines or nodules and a well-produced basal lip of the aperture. These three forms are spatially separated: form A occurs in the Kwae Noi drainage (W5-7), form B in an affluent of the Kaek in Yaeng (K13), and form C in the Kaek River. Comparisons of specimens show that specimens of form B are similar to juveniles of form C. In addition, a graphical chart of shell heights and breadths confirms that forms B and C exhibit a congruent correlation between shell height and breadth (Fig. 8). We conclude that both forms are conspecific and that form B represents predominantly juvenile and sub-adult specimens. In contrast, form A displays a different height–breadth ratio. Together with the distinct sculpture, this is most likely indicative of the fact that the forms A and (B + C) represent two distinct species. Shells of form A resemble the type specimens of *Melania binodosa* Blanford, 1903 more closely than the specimens of form B and C. Showing a similar overall shape, the typical form A of *B. binodosa* differs from *B. armata* essentially by its much larger size. The Kaek River form of *B. binodosa*, however, differs with respect to its more elongated shape, the presence of more whorls, and larger size.

Unlike the former species, *B. microsculpta* has a smooth shell that lacks any sculptural elements except for faint growth lines. Shells comprise one to three whorls, of which the body whorl is the largest, being well rounded to slightly flattened in diameter. The body whorl of some specimens is keeled below the periphery; in others, it is rather rounded. The aperture is broadly ovate, laterally rounded to slightly angulated, and narrowly pointed above. The operculum is round and smaller than the aperture. There has been some confusion with regard to the identity of this species due to a possible mix-up of specimens. Glaubrecht and Köhler (2004) depicted a shell of *B. solemiana* allegedly found at the Sri Dit rapids
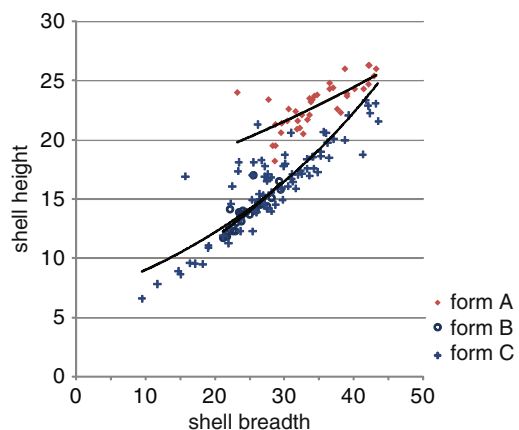

Fig. 8 Diagram showing the relationship of shell height and shell breadth in the three forms of *B. binodosa* recognised by their shape

(K11) and concluded that *B. solemiana* occurs in the upper course of the Kaek River. However, newly collected specimens from the same locality differ from the depicted specimen. They are more similar to the holotype of *B. microsculpta*, which itself is a not very representative specimen of this species due to its exceptionally large size and the rarely found presence of three complete whorls. Here, we correct the former statement that *B. solemiana* occurs in the headwaters of the Kaek River and attribute the relevant specimens from the upper course of the Kaek River at Sri Dit (K111) to *B. microsculpta* instead. This species is found throughout the river between Kaeng Song (K3) and Sri Dit (K11) and also in the affluent creek at km37 (K2).

Each of the three other species with smooth shells, *B. paludiformis*, *B. subgloriosa*, and *Brotia* sp nov3, is only found at a single locality.

Brotia paludiformis has a pronouncedly globular shell that comprises a maximum of two whorls; the second whorl being always much smaller than the body whorl. The aperture is widely oval. The operculum is oval and slightly smaller than the aperture. The species is found only at the Kaeng Sopha waterfalls (K8).

Brotia subgloriosa has been described from the Huai Chieng Nam, a tributary of the Kaek River, where it has not been found since. However, the species also occurs in the upper course of the Kaek River between Sri Dit (K11) and Thung Salaeng (K10). The shell is elongately turreted, relatively large and smooth. The operculum is slightly oval to almost round. *Brotia* sp. nov3 was found at the Aeng Gaw waterfall (K7), which is located near the Kaek River along the course of a small affluent river. Specimens were collected at the end of the rainy season in the flowing water. The creek is not permanent, however, and usually dries out in the dry season. In this period of time, the river is believed to flow only through subterranean cavities in the limestone rocks, where the snails apparently live most of the year. Their body is entirely white (whereas all other known *Brotia* species are brownish or blackish), possibly due to their largely subterranean lifestyle. The shells of this species are rather small, smooth, elongately conical, and comprise three to four well-rounded whorls. The operculum is almost round.

7   Radular Morphology and Substrate Usage

In general, three distinct radula types can be found among the species in the Kaek River (Glaubrecht and Köhler 2004). Type 1 corresponds to the general radular morphology found in a large number of *Brotia* species (see Köhler and Glaubrecht 2006) (Fig. 9a–b, g–h). The radula has a length of around 20–25 mm length (equivalent to about half of the shell height) with about 180–200 rows of teeth (∼9–10 rows/mm). The central teeth have a squarish shape and possess a well-developed glabella and a large main cusp, the lateral teeth have short lateral extensions, and the hooked marginal teeth are moderately long with two cusps – the outer one being broad and ovate in shape, the inner one being much smaller in size. This radula type is found in *B. armata* (Fig. 9a–b), *B. binodosa*,
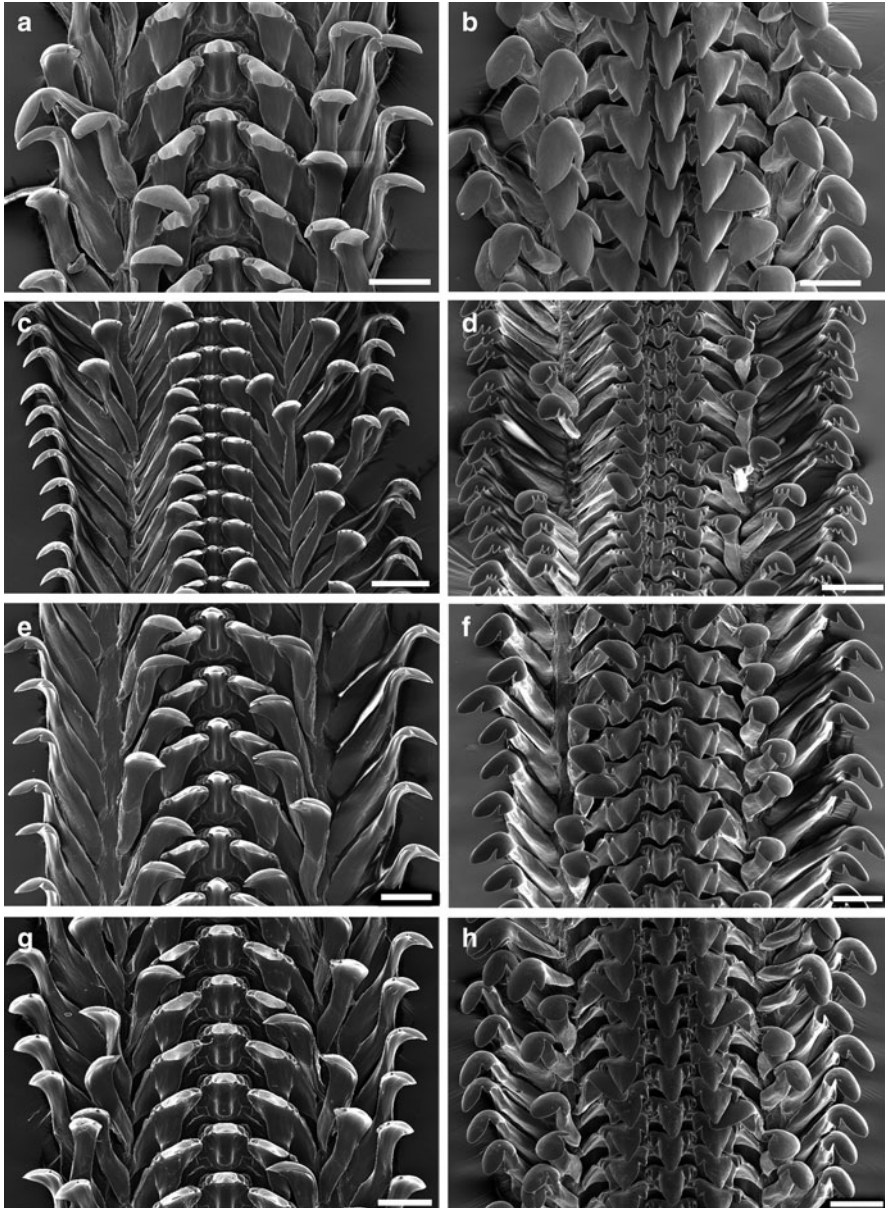
Fig. 9 Representative radulae of *Brotia* species from the Kaek River. Shown are two views of each the same radula segment: *Left*, view from above; *right*, front view at ~45° obliquely from above. (**a,b**) *Brotia armata* (ZMB 114.009, Kaeng Song, on rock; radula type 1). (**c,d**) *Brotia microsculpta* (ZMB 114.223, Poi, on rock; type 3). (**e,f**) *Brotia microsculpta* (ZMB 114.054, Sri Dit, on rock; type 2). (**g,h**) *Brotia subgloriosa* (ZMB 114.046, Thung Salaeng, on sand; type 1). *Scale bars* 100 μm

Table 2 Radula types found among *Brotia* species in the Kaek River. Given are means and standard deviations of the length of the radular ribbon (mm), numbers of rows of teeth, and rows per mm of ribbon length

Species	Examined radulae	Radular length	Rows of teeth	Rows per mm
Type 1				
B. armata	31	21.5 ($\pm$4.0)	194 ($\pm$33)	9.1 ($\pm$1.1)
B. binodosa	18	19.6 ($\pm$4.7)	189 ($\pm$32)	9.8 ($\pm$2.0)
B. paludiformis	1	23.2	178	7.7
B. pseudosulcospira	3	25.1 ($\pm$3.2)	224 ($\pm$28)	8.9 ($\pm$0.2)
B. subgloriosa	10	17.6 ($\pm$4.1)	185 ($\pm$25)	10.8 ($\pm$1.9)
Type 2				
B. microsculpta (2)	13	16.7 ($\pm$4.1)	182 ($\pm$38)	11.2 ($\pm$2.1)
Type 3				
B. microsculpta (3)	4	12.6 ($\pm$4.7)	220 ($\pm$86)	17.7 ($\pm$2.2)
B. sp. nov3	2	14.1 ($\pm$2.1)	214 ($\pm$18)	15.3 ($\pm$1.0)

B. paludiformis, *B. pseudosulcospira* and *B. subgloriosa* (Fig. 9g–h) without marked and consistent interspecific differences. Type 2 is similar to the former type but differs by the presence of a narrower and shorter glabella of the central tooth, a slightly shorter ribbon length, and more densely packed rows of teeth (Fig. 9 e–f; Table 2). These conditions have been found in 13 of 17 examined specimens of *B. microsculpta*. Type 3 is rather distinct and differs from the others by a generally much shorter ribbon length (~15 mm), significantly more densely packed rows (~15–17 rows/mm), a more weakly developed glabella of the central teeth, lateral teeth with longer lateral extensions, and marginal teeth with a more elongated shape that support two or three accessory inner cusps (Fig. 9c–d). Type 2 has been found in 4 of 17 specimens of *B. microsculpta* and in the two examined specimens of *Brotia*. sp. nov3.

Radulae of the types 1 and 2 are rather similar, and the differences between them are subtle with the deviant shape of the glabella of the central tooth being the only distinctive feature. In addition, some radulae of *B. armata* have been found to also possess relatively short and narrow glabellas representing transitional stages between the two types. Type 2 has also been reported from *B. solemiana* by Glaubrecht and Köhler (2004) both from in and outside the river (but note that this name was erroneously attributed to specimens of *B. microsculpta* from the upper course of the Kaek River). This observation has been confirmed by the present study with respect to *B. solemiana* from the Loei River drainage.

Our recent findings are also in agreement with the observation of Glaubrecht and Köhler (2004) that *B. microsculpta* has the most distinctive radula among the Kaek River species (together with the newly found *Brotia* sp. nov3 from the Aeng Gaw waterfall).

During field work in 2006 and 2007, sandy and muddy areas as well as rock surfaces were systematically searched for specimens. In general, all *Brotia* species were found to graze on rocks irrespective of which radula type they possess, whereas sand and mud flats were as a rule not found to be inhabited by *Brotia*

species. Only exceptionally, single individuals of different species were found on sand (while no *Brotia* was ever found on mud), which suggests that these snails do usually not live on this substrate but occur there by accident. Only at the Thung Salaeng rapids, were specimens of *B. subgloriosa* frequently (but not exclusively) found on small sandy patches within rock holes formed by scouring. Next to *B. subgloriosa*, individuals of *B. binodosa* were also found in these holes. These holes had limited surface areas (usually not more than ~0.5–5 m^2), however, and in order to reach them, snails would have needed to crawl over larger stretches of rock.

In summary, our recent findings do not indicate that there is a correlation between radula morphology and substrate usage in *Brotia* species in the Kaek River. First, there are no obvious differences between type 1 radulae of specimens collected on rock and sand. Second, the species with the most distinctive radulae, *B. microsculpta* and *Brotia* sp. nov3, do not differ from any other species in the way they utilise a certain substrate. In contrast, at various sites, *B. microsculpta* was found to occur syntopically with other rock grazers, such as *B. armata*. This result contradicts earlier assumptions by Glaubrecht and Köhler (2004) that there is a possible correlation between radula phenotypes and environment (substrate) among species of the Kaek River flock, which was based on limited observations.

In order to infer phenotypic responses to changing substrates, we set up aquarium experiments than ran over the period of 1 year between May 2007 and April 2008. In order to test if grazing on different substrates affects the radular morphology, we conducted transplant experiments. Series of 10–12 individuals each of *B. armata*, *B. binodosa*, and *B. microsculpta* collected on rocky surfaces and of *B. binodosa* and *B. subgloriosa* collected on sand were split into two groups. Each group was kept for the entire period in aquaria that provided either only sand or only rocks as substrate. Under both settings, animals were fed with fish food and various kinds of vegetables. After the period of 1 year, the radulae of these animals (and their young) from different aquarium set-ups were compared with each other as well as with specimens collected at the same localities in the wild. The numbers of compared radulae were low as some specimens died during the period of study. Radulae of some individuals that were born and raised in the aquaria were not analysed since their intermediate shell phenotypes suggested that they were of hybrid origin, which might have also affected the radular morphology. In fact, the shell morphology of these specimens that grew up in the aquaria corresponded with *B. armata* or *B. binodosa*, while they had radulae of type 2 normally being found in *B. microsculpta*.

For the low numbers of compared radulae of captive adults ($n = 14$), we refrained from a statistical analysis of our results. However, in general, the specimens raised in aquaria (including both adult animals collected in the field as well as most specimens that were newly born in the aquaria) showed no significant changes in their dentition patterns (by means of the shape of teeth) with respect to specimens collected at the same localities in the wild irrespective of the substrate on which they were kept.

Radulae of captive animals only differed from those collected in the field by having slightly shorter ribbons (in average by 1–2 mm shorter), while the density of

rows did not vary significantly with respect to specimens collected in the wild. Independent of the substrate provided, the habitat conditions in the aquaria certainly differed from those in the native environment. For example, in the field, *Brotia* species were found to graze on the biofilm on hard substrates, while in the aquarium, the main source of food was fish food. Since these altered habitat conditions did not result in significantly changed radular dentition, we conclude that in *Brotia* the phenotypic plasticity of the radula with respect to the source of food or the utilised substrates is rather limited. This finding contrasts with reports of immense intraspecific variation observed in rock-dwelling Littorinidae, which was partly attributed to phenotypic plasticity (Padilla 1998; Reid and Mak 1999). The observed inconsistency underscores the need for further studies that address the plasticity of the radula in different gastropod groups with respect to ecological conditions.

8 Phylogenetic Relationships Inferred by Analyses of Mitochondrial Genes

In order to infer the phylogenetic relationships of the Kaek River species, a partial fragment of the cytochrome c oxidase gene (COI) was analysed by employing Bayesian Inference. Previous studies based on analyses of combined 16S and COI data have already demonstrated the monophyly a Central Thailand clade of *Brotia*, which comprises all species inhabiting the drainages of the Kaek, Kwae Noi, Loei, Pa Sak, and Pong Rivers in northern Central Thailand (Köhler and Glaubrecht 2006; Köhler and Dames 2009). An earlier study has also suggested the monophyly of the Kaek River species flock based on 16S and COI (Glaubrecht and Köhler 2004). Compared to these previous studies, the phylogeny reconstructed here is based on a significantly more comprehensive basis of data with respect to both taxon sampling and area covered. The previous study of Glaubrecht and Köhler (2004) did not include species from the Pa Sak and Loei drainages and only one sample each from the Pong and Kwae Noi drainage. A comprehensive coverage of the pachychilid fauna of all five rivers, however, is required if we want to understand the evolution of the Kaek River species flock, due to the geological history of the entire area which has seen altered flow regimes of rivers as explained above. The present phylogeny has been computed with *Brotia sumatrensis* used as outgroup because it was found by Köhler and Glaubrecht (2006) to be the sister group of the Central Thailand clade. The outgroup has subsequently been pruned from the tree.

In general, the phylogenetic tree (Fig. 10) has a very flat topology, and species recognised by their morphology fall not into monophyletic clusters but remain widely unresolved. However, the tree contains largely monophyletic, drainage-specific clades. Species from the Kaek River form a huge monophyletic crown-group, which includes admixed sequences of *B. armata*, *B. binodosa* and *Brotia* sp.
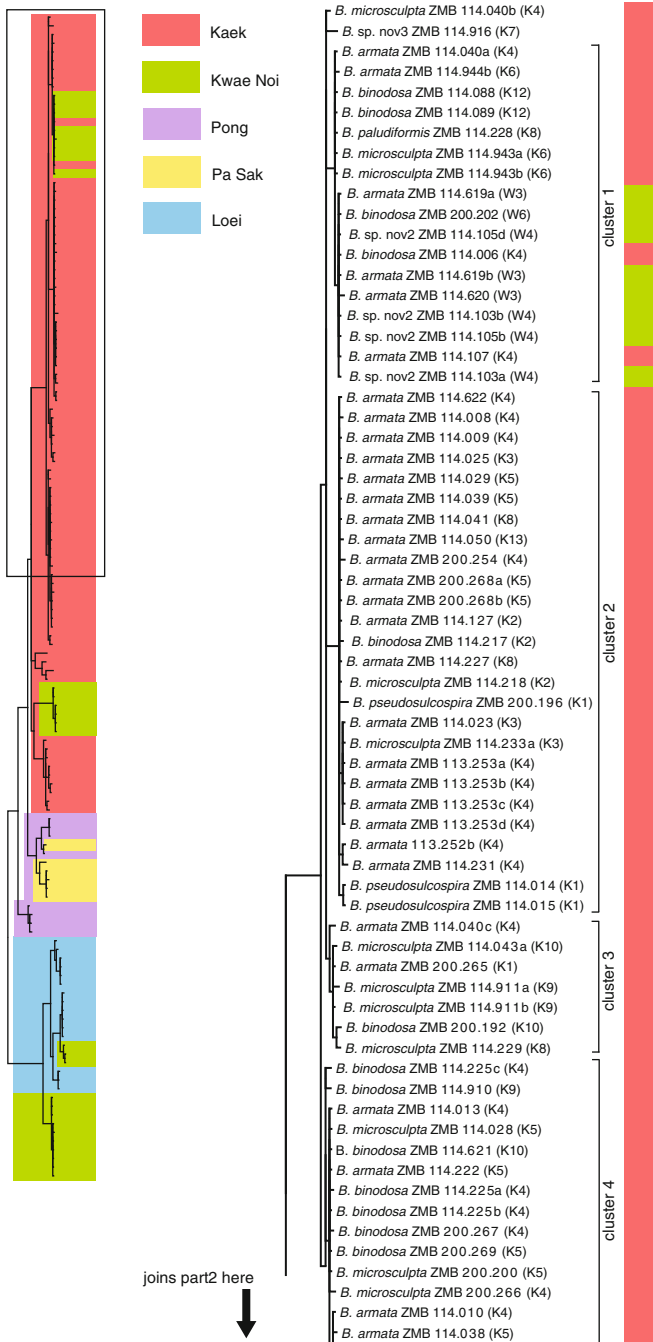
Fig. 10 Phylogenetic tree based on analyses of partial sequences of COI showing the relationships among the *Brotia* species from central Thailand as inferred by Bayesian Inference. Outgroup pruned from the tree (part 2, see former page for continuation) Left hand side: entire tree, right hand side: enlarged portion
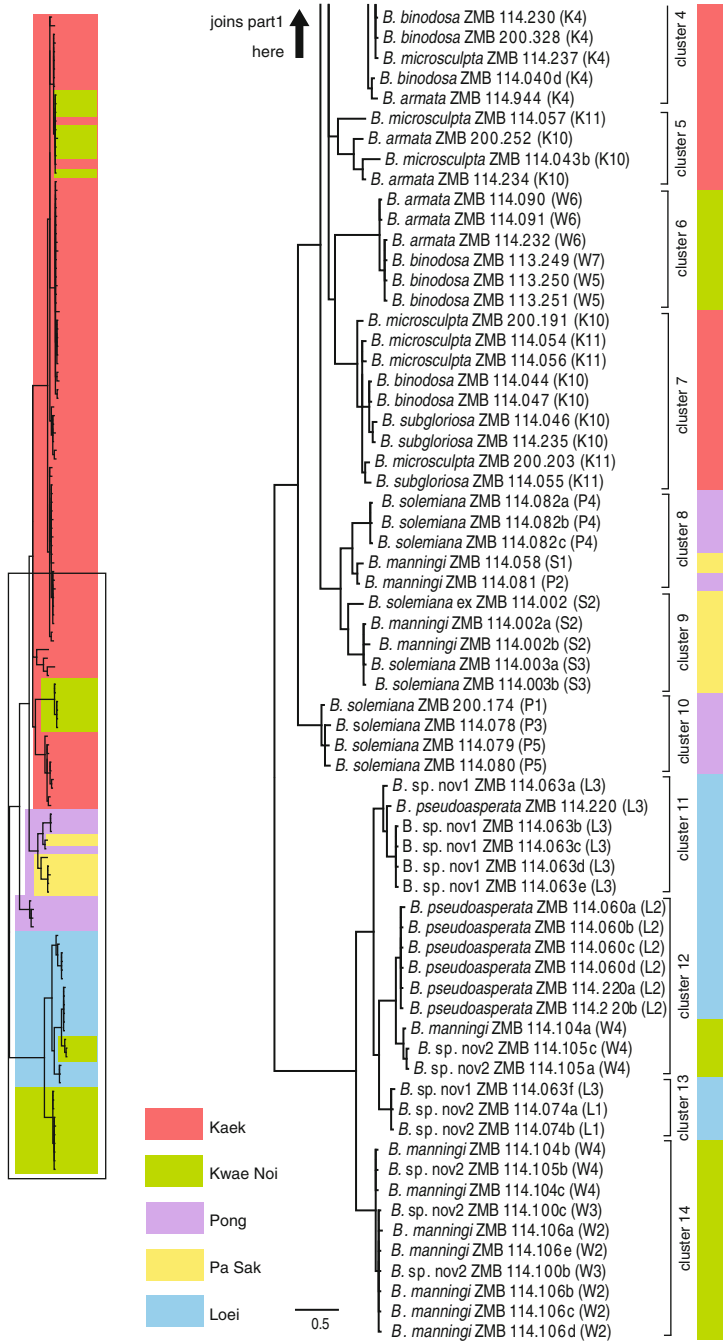
Fig. 10 (continued) (past 2, see forms page for continuation). Left hand side: entire tree, right hand side: enlarged portion

nov2 from the Kwae Noi (Kaek-Kwae Noi clade). Haplotypes from the Pong and Pa Sak River form a second clade (Pa Sak-Pong clade), which is the sister group of the Kaek-Kwae Noi clade. A further clade exclusively contains Pong River-specific haplotypes (Pong clade) and forms the sister group of the two previous clades together (Kaek-Kwae Noi + Pa Sak-Pong). Eventually, at the most basal bifurcation of the tree, a clade containing haplotypes from the Loei and Kwae Noi Rivers (Loei-Kwae Noi clade) forms the sister group of all previously mentioned clades. All aforementioned river clades are well-differentiated from each other by means of average genetic distances between around 5 and 12% (Tables 3 and 4).

The Kaek-Kwae Noi clade comprises seven more or less well-differentiated haplotype clusters as well as two single sequences that do not cluster together with any of the others. These sequences may represent another rare (or rarely sampled) haplotype cluster. The mean sequence divergence within the clusters does not exceed a maximum of 1.5% while the divergence between clusters ranges between 1.3 and 7.5% (Table 3).

The three haplotype clusters at more basal positions of the Kaek-Kwae Noi clade (5–7) are especially well differentiated and contain only sequences of specimens collected at upstream localities (K10–K11) or in the Kwae Noi drainage (W5–W7), while the haplotype clusters at more derived positions (1–4) show little genetic differentiation overall and contain mostly specimens collected at midstream locations (K1–K6), but also admixed sequences from upstream localities (K10–K11) and the Kwae Noi drainage (W3–W6). In addition, there is one well-differentiated

Table 3 Mean sequence divergence within and between the haplotype clusters 1–7 of the Kaek-Kwae Noi clade (in %, Kimura-2-parameters)

Cluster	1	2	3	4	5	6	7
1	0.1						
2	0.2	0.2					
3	1.3	1.7	0.4				
4	1.4	1.8	1.1	0.4			
5	4.1	4.7	4.1	4.4	1.5		
6	6.4	7.5	7.0	6.7	5.8	0.2	
7	4.8	5.6	5.1	4.9	4.3	5.6	0.6

Table 4 Mean sequence divergence within and between haplotype clusters and phylogenetic clades from different drainage systems (in %, Kimura-2-parameters model). Bold frames: sequence divergence within drainage-specific clades

Clade	Kaek- Kwae Noi	Pa Sak-Pong			Pong	Loei-Kwae Noi		Kwae Noi
Cluster	1–7	8	9	10	11	12	13	14
1–7	0.1–7.5							
8	5.8–6.9	1.1						
9	4.9–6.1	3.3	0.9					
10	5.8–11.2	5.7	5.7	0.5				
11	9.5–11.2	9.5	10.7	10.9	0.5			
12	9.6–11.7	9.2	10.1	10.5	3.2	0.4		
13	10.2–12.2	9.5	11.1	11.0	2.6	2.6	0.1	
14	9.1–11.4	9.1	10.0	10.1	4.4	4.1	4.1	0.1

haplotype cluster (6) that exclusively contains sequences of *B. armata* and *B. binodosa* from the Kwae Noi drainage.

Within the Pa-Sak-Pong clade, there are three distinct haplotype clusters that are separated from each other by genetic distances between 3.3 and 5.9%, while the genetic differentiation within the clusters does not exceed 1.1% (Table 4). The Loei-Kwae Noi clade contains two clusters with 2.6% sequence divergence. In addition, there is an additional, well-differentiated river-specific cluster within each of the Pong and Kwae Noi River clades (Fig. 10; Table 4).

Among *Brotia*, interspecific distances in COI between morphologically well-differentiated and allopatric species were frequently found to be as low as 1.4–1.7% (Köhler and Glaubrecht 2006). This value is considered to represent a conservative estimate of a minimum threshold for interspecific rates of sequence divergence in this group. Compared to this threshold, the amount of genetic differentiation within the Central Thailand clade of *Brotia* would be equivalent to the existence of at least 12 distinct (species-specific) gene pools (or 13 if the 2 single sequences are considered that do not cluster together with others). Accordingly, within the Kaek-Kwae Noi clade there are between 5 and 6 such distinct groups (clusters $1 + 2, 3 + 4, 5, 6, 7$, single sequences) plus 3 within the Pa Sak-Pong clade, 2 within the Loei-Kwae Noi clade, and 1 each within the Pong and Kwae Noi clades, respectively. This number of genetically well-differentiated groups correlates perfectly with the total number of 13 species as recognised by their morphology (*B. armata*, *B. binodosa*-A, *B. binodosa*-B, *B. manningi*, *B. microsculpta*, *B. paludiformis*, *B. pseudoasperata*, *B. pseudosulcospira*, *B. solemiana*, *Brotia* sp. nov1, *Brotia* sp. nov2, *Brotia* sp. nov3, *B. subgloriosa*) (see Fig. 11 for photographs of living specimens of some of these species). In addition, there is also a good correlation between the numbers of genetically distinct groups and the numbers of recognised "morphospecies" in each river drainage (genetic group/morphospecies): Kaek River (6/7), Kwae Noi (3/4), Loei (3/3), Pong (2/2), Pa Sak (2/2).

However, generally, there is a significant mismatch between the branching of the mitochondrial gene tree into haplotype clusters or groups and the distribution of morphologically recognised species across this topology, which apparently renders all morphospecies as polyphyletic assemblages (Fig. 10). The average infraspecific genetic distances among most of these morphospecies exceed by far the empirical minimum threshold of interspecific differentiation as mentioned above (with values up to 5.8%) and lie well within the range of observed interspecific genetic distances of 1.8–10.7%.

9 Towards an Evolutionary Explanation: Conclusions from Incongruence

Other studies of cerithioidean freshwater gastropods have also revealed in part extensive incongruence between the branching patterns of mitochondrial gene trees and the delineation of (putative) species by use of morphological characters
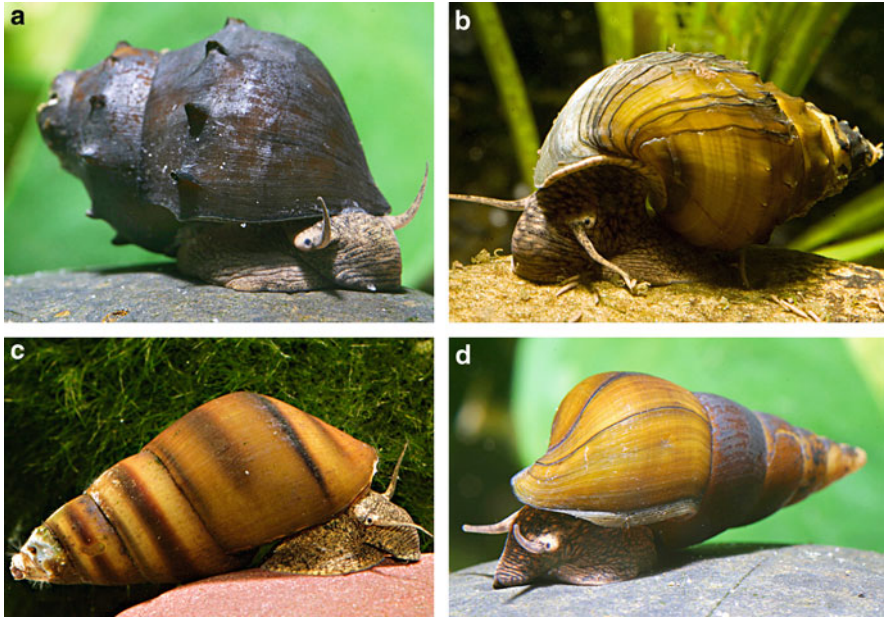
Fig. 11 Photographs of living specimens. (**a**) *Brotia armata*. (**b**) *Brotia binodosa*. (**c**) *Brotia manningi*. (**d**) *Brotia subgloriosa*. Photos (**a**,**d**) courtesy of Chris Lukhaup (Bittenfeld), photos (**b**,**c**) courtesy of Andreas Helmenstein (Gummersbach)

(Lydeard et al. 2002; Minton and Lydeard 2003; Wilson et al. 2004; Köhler and Glaubrecht 2006; Lee et al. 2007; Rintelen et al. 2007; Glaubrecht and Rintelen 2008; Köhler and Dames 2009; Köhler et al. 2009; Strong and Köhler 2009). Workers attributed these discrepancies between gene and species trees to a wide range of phenomena, including incomplete lineage sorting, the existence of cryptic species, taxonomical over-splitting of lineages, and hybridisation. However, strikingly different conclusions were drawn from quite similar observations depending on which actual cause has been postulated. For example, in Korean *Semisulcospira* populations (Semisulcospiridae), both mitochondrial (16S) and nuclear (28S) genes of seven species recognised by their morphology (*S. libertina*, *S. coreana*, *S. forticosta*, *S. gottschei*, *S. multicincta*, *S. nodiperda*, *S. tegulata*) revealed a structure that followed geographical rather than taxonomic trajectories, with haplotypes and genotypes largely clustering into drainage-specific clades, but without resolution with respect to morphologically delineated species (Lee et al. 2007). The authors concluded that pending the demonstration of any reliable differentiation within this complex, all but one species should be synonymised into a single polymorphic species complex – *S. libertina*. In contrast, faced with similar phenomena in the pachychilid taxon *Tylomelania*, Rintelen et al. (2004) and Glaubrecht and Rintelen (2008) suggested that incomplete lineage sorting and introgressive hybridisation caused the mismatch of gene and species trees, and

concluded that mitochondrial gene trees are misleading with respect to the recognition of species. In general, we agree that the pervasiveness of this phenomenon across various freshwater cerithioidean groups, in combination with the specifics of mitochondrial DNA inheritance (Nichols 2001; Funk and Omland 2003; Ballard and Whitlock 2004; White et al. 2008), corroborate the notion that mitochondrial markers may have limited utility in assessing status at the species level, and that a meaningful molecular characterisation of species should make use of a combination of mitochondrial and fast evolving nuclear markers (in addition to morphology, of course). We anticipate that amplified fragment length polymorphism (AFLP) or microsatellite analyses are the methods of choice to address species limits within the present group, since both methods are known as powerful tools to resolve relationships at the population to species-level and to investigate the gene flow between populations (see, e.g., Richard and Thorpe 2001; Albertson et al. 1999; Savekoul et al. 1999). The application of AFLP analyses is part of the present research project; the completion of the analyses is pending.

Nevertheless, even in the absence of comparative analyses of nuclear markers, the patterns of morphological and mitochondrial differentiation provide intriguing insights into the evolution of the Kaek River species flock. As mentioned above, in principle, several phenomena may explain the mismatch between gene and species trees in the present case. We exclude the possibility that the incongruence is caused by nuclear pseudogenes ('numts'), because translation of the analysed COI sequence alignment into amino acid sequences produced a highly conserved alignment that did not contain stop codons or gaps. We also do not consider ancestral polymorphism as a possible explanation for incongruence because the observed rates of sequence divergence of up to 1.5% within and 5.8% between haplotype clusters are considered to be out of the range of infraspecific polymorphism. Ancestral polymorphism may only be considered as an explanation for the unresolved relationships within sub-clades that overall show low rates of genetic differentiation, such as haplotype clusters 1–4. In contrast, the presence of morphologically cryptic species cannot be entirely ruled out as a possible cause for apparently unresolved species limits. The presence of potentially misidentified (=cryptic) species could indeed explain why morphologically similar populations from different river drainages, such as those attributed to *B. manningi* or *Brotia* sp. nov2, appear at different positions in the tree, or why Kaek River species, such as *B. microsculpta*, seem to have two different radula types. However, this explanation is very unlikely to account for genetic admixtures among morphologically distinctive species within a single drainage system, such as *Brotia* sp. nov1 and *B. pseudosulcospira* in the Loei drainage or *B. manningi* and *Brotia* sp. nov2 in the Kwae Noi drainage. In these cases, the low rates of genetic differentiation contradict the presence of further unrecognised species. Furthermore, morphological shell polymorphism (e.g. ecophenotypism) is also unlikely to account for the unresolved species limits. It has been demonstrated that modifications of shell sculptures may occur in freshwater cerithioideans depending on the substrate (Urabe 2000). However, it has also been demonstrated that ecophenotypism is restricted to

relatively small changes while clear differences (as found here) are considered to be genetically controlled (Gittenberger et al. 2004; Haase and Misof 2009).

We are convinced that introgressive hybridisation caused by cross-breeding is the most likely cause for a great deal of the observed incongruence between the mitochondrial gene and the morphological species tree – however, this is a hypothesis that can only be validated by comparative analysis of genetic markers from other linkage groups (i.e. nuclear genes). In fact, various studies of land snails have shown that introgressive hybridisation, though difficult to demonstrate conclusively, accounts for unresolved species limits in mitochondrial gene trees (Thacker and Hadfield 2000; Goodacre and Wade 2001; Haase et al. 2003; Haase and Misof 2009), and similar conclusions were drawn for pachychilid freshwater gastropods (Glaubrecht and Rintelen 2008; Köhler et al. 2009). In most of their range across South and Southeast Asia, *Brotia* species have restricted distributions, being confined to the headwaters of single rivers or creeks but absent from the lower courses of larger streams. There are usually two species at the most that co-occur in a given habitat while the majority of species occurs in allopatry or parapatry. Accordingly, Köhler et al. (2009) suggested that geographical separation is the main factor that drives speciation in pachychilids in the rivers of mainland Asia, and that, when no isolation mechanisms have evolved that prevent species from cross-breeding, secondary contact between originally allopatric populations or species frequently leads to the introgression of neutral markers. In agreement with this hypothesis, the more or less random distribution of morphotypes across drainage-specific haplotype clades is probably best explained by introgression of mitochondrial genes into foreign gene pools due to secondary contact of previously isolated populations or species caused by the translocation of specimens either due to dispersal or vicariance events. Indeed, this assumption agrees well with theoretical considerations, which predict that foreign invasions of already occupied territories lead to massive introgression of neutral genes if interbreeding is not severely prevented between invading and local species. In such cases, introgression occurs almost exclusively from the local to the invading species, especially for populations located far away from the source of the invasion, and this occurs irrespective of the relative densities of the two species (Currat et al. 2008). It has also been argued by the authors that this pattern is strongest in markers experiencing reduced gene flow, which implies that organelle genes are often preferentially introgressed across species boundaries. Such massive introgression has the potential to explain the observed rates of discordance in the COI tree presented here. In addition, we believe that the presence of two different radula types in *B. microsculpta* can be attributed to the existence of species hybrids. *Brotia microsculpta* exhibits a very distinct radular morphology (type 3), while type 2, which is somehow an intermediate form between types 1 and 3, is possibly found in hybrids. Because most *Brotia* species have radulae of the generalised type 1 anyway, their hybrids cannot be recognised by the radula morphology.

10   Dispersal or Vicariance: Genetic Exchange Between River Faunas and the Relevance of River Captures Within the Mekong Drainage System

Above, we have postulated that massive introgression of haplotypes occurred due to extensive faunal exchange across the five river drainages studied herein. We were interested to learn whether there are corresponding patterns in the timing of geological events in the region (i.e. river captures) and the occurrence of major splits in the phylogenetic tree. We performed a likelihood ratio test in order to test whether our sequence data would allow for a molecular clock approach. However, a chi squared test showed that Bayesian trees produced under the conditions of a strict clock resulted in significantly lower likelihood scores compared to an analysis in which branches were allowed to evolve at variable rates. The application of a molecular clock under use of an external calibration as suggested by Wilke (2003) for our COI data was therefore refuted. It was thus not possible to test whether certain splits in the tree fall within the time frame of major tectonic events in Central Thailand.

The fossil record of *Brotia* dates back as far as Middle Miocene (Annandale 1919; Gurung et al. 1997), which is equivalent to a minimum age of the entire group of at least 8–12 Ma. Even though the Central Thailand clade is found at a derived position in the molecular tree, it is plausible to assume that it has originated several million years ago. Accordingly, we postulate that the era from the late Pliocene to the Quaternary was critical for the evolution of the gastropods under study. This was a time when stream captures of various magnitudes impacted river alignments in northern Central Thailand as a result of local tectonic or hydrological processes (see above). Probably the most important event was the realignment of the Mekong River, which between ∼1 and 0.05 mya flowed through the Loei-Pa Sak river beds. Because *Brotia* species do usually not inhabit the mid- and upstream regions of larger rivers, changes of the flow direction of the Mekong may have both connected populations in earlier stages of the realignment of stream, when the flow regime has been at a lower magnitude, and separated populations, when the Mekong formed a large stream which was not a suitable habitat for *Brotia*. For instance, it is considered possible that the disjunctive distribution of *B. manningi*, which occurs in both the Pa Sak and Kwai Noi drainage, may have been caused by the realignment of the Mekong. Genetic exchange between river drainages may have occurred either due to the translocation of specimens from one river to the other (dispersal) or due to events related to the geological history of the area (vicariance). The importance of dispersal is difficult to both reject or confirm. However, we believe that tectonic events and processes since the mid-Tertiary have likely influenced the evolution and distribution of species by mediating phases of contact and isolation of faunas through the capture or separation of river systems. Attwood and Johnston (2001) have shown that episodic changes of river catchments have had a significant influence on the distribution and evolution of pomatiopsid snails by separating and reconnecting populations or species. There is little doubt that other freshwater

animals with low dispersal abilities, such as pachychilid gastropods, may also have been affected by these changes. However, while the mtDNA tree provides information on the divergence of clades, it tells us little about gene flows between drainage-specfic clades because introduced foreign haplotypes become quickly replaced by local, drainage-specific haplotypes due to the general directionality of introgression from local to alien species (Currat et al. 2008). Therefore, unlike divergences, events that connected river faunas are difficult to trace in the mtDNA-based phylogeny. Gene flows across the borders of drainage systems can probably be confirmed only if they have occurred more recently, because then the foreign haplotypes may not yet have been completely replaced by local ones. This seems to be the case in the phylogenetically derived Kaek River-specific haplotype cluster 1, which also contains specimens from the Kwae Noi drainage. The low genetic differentiation suggests that the underlying gene flow between the Kaek and Kwae Noi Rivers must have occurred rather recently.

11   Speciation and Radiation of *Brotia* in the Kaek River

The *Brotia* species flock in the Kaek River shows some unique aspects that call for an explanation. The number of *Brotia* species occurring in the river exceeds that found in any other river across SE Asia by at least two times. Additionally, these species live largely in sympatry whereas species in other drainages mostly occur in different sectors or tributaries in complete spatial isolation or with only narrow zones of contact. The dense sampling regime covering the entire region of northern Central Thailand has revealed that all but one species (*B. armata*) are indeed endemic to the Kaek River and, consequently, must have evolved within the drainage system. Glaubrecht and Köhler (2004) argued that the lack of resolution in the molecular phylogeny and its shallow topology indicate the recent origin of the Kaek River species flock and, consequently, a rapid morphological divergence of its constituent species. Preliminary results have suggested that the Kaek River species flock may have evolved as a result of an adaptive radiation and that ecological factors may have driven speciation. It has been the foremost goal of the present study to test this hypothesis. Streelman and Danley (2003) suggested for vertebrates that radiations usually follow similar evolutionary trajectories. Groups diverge along the axes of habitat and trophic morphology as well as communication, often in that order. They argued that divergence with respect to habitat and trophic morphology is likely to follow ecological selection models and that divergence with respect to communication proceeds according to sexual selection models. In agreement with this postulate, studies of the confamiliar gastropod genus *Tylomelania* have shown that indeed substrate choice and trophic specialisation seem to trigger speciation. It remained to be tested if corresponding patterns were to be found in the Kaek River species flock. However, herein we show that the *Brotia* species in the Kaek River do not differ with respect to their preferred substrate or the water depth at which they were found. The radular dentition of

most species is very similar, and only two species differ clearly from all others by possessing a distinct radula type (*B. microsculpta*, *Brotia* sp. nov2). Both findings suggest that habitat-mediated segregation and trophic specialisation have not played a significant role in the evolution of the Kaek River species flock. In addition, it is unlikely that speciation has been triggered by sexual selection due to the likely presence of species hybrids, which hints towards incomplete mechanisms of postzygotic isolation. Consequently, to our current knowledge, there is no evidence in favour of the assumption that ecological speciation has accounted for the diversity of species in the Kaek River. Alternatively, speciation within the Kaek River is currently best explained by geographical isolation. Firstly, it cannot be ruled out that some species originate from outside the Kaek River and that introgressed Kaek River-specific mtDNA has replaced the foreign haplotypes, by obscuring traces of repeated river colonisation (see Currat et al. 2008). Secondly, it is suggested by the mtDNA-based phylogeny that gene flow within the Kaek River occurs predominantly from upstream to downstream areas because basal haplotype clusters belong exclusively to upstream populations whereas derived haplotype clusters contain a mixture of both up- and midstream populations. The Kaek River flows over a series of waterfalls and cascades. Although according to own observations snails are able to crawl above the water line and may thus in principle be able to climb the vertical walls, the waterfalls seem to form barriers that significantly delimit the gene flow across vertical structures against the direction of flow. In addition, the water regime of the Kaek River is rather unstable. The ground, consisting predominantly of sandstone and limestone, is very permeable for water, which may cause large sectors of the river to fall dry during extended periods of drought. Even in regular dry seasons, the water body of the Kaek River is largely reduced and some of its affluents become entirely dry (Fig. 4f).These fluctuations regularly cause local extinctions in restricted stretches of the river and its affluents, which are followed by re-colonisation of the areas in the rainy season. Both factors, river fragmentation by waterfalls and regular local extinctions, may assist the retention of a reticulate genetic structure and the conserving of rates of local genetic differentiation. Moreover, extended periods of drought may have occurred during the Cenozoic and could have triggered speciation in peripheral isolates – a process generally considered as a significant modus of allopatric speciation (Mayr 1963). It has been shown for plants that small populations may differentiate quickly (Ellstrand and Elam 1993), and the limitations for gene flow as described above may have assisted this genetic differentiation to persist. In analogy, it has been confirmed by theoretical considerations that rapid parapatric speciation on the time scale of up to a few thousand generations is plausible even in the presence of moderate genetic exchange between neighbouring subpopulations. Divergent selection for local adaptation is also not required for the evolution of reproductive isolation as a by-product of genetic divergence (Gavrilets et al. 2000). The authors showed that populations or species with small range sizes should have higher speciation rates – circumstances that probably do apply in the present case.

Hence, the present model case of a riverine radiation apparently does not follow the same evolutionary trajectories as recently demonstrated for a number

of lacustrine radiations of various groups of animals, which involve a major ecological component (i.e. ecological speciation, sensu Schluter 2000). By contrast, the flock of *Brotia* species in the Kaek River has more in common with other riverine radiations, such as the Triculinae of the Mekong. It has been demonstrated that speciation and radiation in these freshwater snails were triggered by geological events, such as the uplift of mountain chains, lava flows, and river captures or realignments, as well as waves of local extinctions and re-colonisations (Davis 1979, 1981; Attwood and Johnston, 2001) – all of which have probably inititiated speciation in peripheral isolates. Similar patterns have been observed in the Tasmanian hydrobiid *Beddomeia* (Ponder et al. 1993) and the hydrobiid *Fluvidona* in Victoria, Australia, (Ponder et al. 1994). Both radiations involve small-range species. The mode of speciation is allopatric or parapatric and mainly driven by vicariance due to restriction of ranges resulting in isolation and subsequent differentiation of peripheral populations. This does not exclude secondary sympatry of closely related species, such as in *Fluvidona*, which followed events of speciation in isolation due to restrictions of habitats. In summary, we conclude that, with respect to adaptive radiations of freshwater organisms, long-lived lakes provide unique environmental conditions that may facilitate ecological speciation. In contrast, rivers apparently provide different conditions that favour para- and allopatric models of speciation.

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