

Eocene habitat shift from saline to freshwater promoted Tethyan amphipod diversification

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Current theory predicts that a shift to a new habitat would increase the rate of diversification, while as lineages evolve into multiple species, intensified competition would decrease the rate of diversification. We used Holarctic amphipods of the genus *Gammarus* to test this hypothesis. We sequenced four genes (5,088 bp) for 289 samples representing 115 *Gammarus* species. A phylogenetic analysis showed that *Gammarus* originated from the Tethyan region with a saline ancestry in the Paleocene, and later colonized the freshwater habitat in the Middle Eocene. Ancestral range reconstruction and diversification mode analysis combined with paleogeological and paleoclimatic evidence suggested that the habitat shift from saline to freshwater led to an increased diversification rate. The saline lineage of *Gammarus* dispersed to both sides of the Atlantic at 55 million years ago (Ma), because of the few barriers between the Tethys and the Atlantic, and diversified throughout its evolutionary history with a constant diversification rate [0.04 species per million years (sp/My)]. The freshwater *Gammarus*, however, underwent a rapid diversification phase (0.11 sp/My) until the Middle Miocene, and lineages successively diversified across Eurasia via vicariance process likely driven by changes of the Tethys and landmass. In particular, the freshwater *Gammarus lacustris* and *Gammarus balcanicus* lineages had a relatively high diversification shift, corresponding to the regression of the Paratethys Sea and the continentalization of Eurasian lands during the Miocene period. Subsequently (14 Ma), the diversification rate of the freshwater *Gammarus* decreased to 0.05 and again to 0.01 sp/My. The genus *Gammarus* provides an excellent aquatic case supporting the hypothesis that ecological opportunities promote diversification.

evolution | molecular dating | range expansion

Colonization of a new habitat often opens up new ecological opportunities and thus promotes lineage diversification (1–3). This is a major tenet in modern evolutionary biology and there are numerous successful case studies in terrestrial systems, particularly in oceanic archipelagos. Darwin's finches on the Galápagos Islands and the drosophilid flies of the Hawaiian Islands are perhaps the best known cases (4, 5). In aquatic systems, habitat shifts from marine to freshwater have been frequently documented (6); however, whether such habitat shift promotes diversification has not been vigorously tested. Marine and freshwater are very different ecosystems; in addition to a significant difference in salinity, locally adapted coinhabitants provide different resources and competitions (7). To investigate the evolutionary implications of such habitat shift, an appropriate choice of organism group is essential. A speciose group with limited long-distance dispersal ability, occurring in both freshwater and saline water and inhabiting an area with well-known geological history, would be ideal (8).

Amphipod crustaceans of the genus *Gammarus* seem to satisfy these criteria. The genus includes over 200 described species and is distributed across the entire Holarctic. Although the majority inhabit freshwater, a large number of species occur in amphiatlantic, Mediterranean, and Ponto-Caspian saline water systems (9). Early phylogenetic work suggested a possible habitat shift from marine to freshwater (10). Amphipods lack an in-

dependent larval dispersal stage, and all freshwater species except *Gammarus lacustris* are endemic to restricted areas. The current disjunctive distribution of *Gammarus* indicates that the evolutionary history of this group may bear a clear signature of past geological events, such as the plate tectonic effects and the Tethyan events described below.

In the Late Eocene [37–34 million years ago (Ma)], the collision between India and Asia promoted the uplift of the Tibetan plateau, which disrupted the exchange between the Tethys Ocean and the Eastern Asian water systems (8, 11). The convergence between Africa and Europe formed an active Alpine mountain belt along the southern border of the Eurasian continent, which isolated the Paratethys Sea from the Mediterranean Sea (11). The Paratethys existed as far east as Tajikistan and was connected with the Arctic Ocean and the North Sea by the Turgai Strait and the Danish-Polish trough (12, 13). These two seaways divided the Eurasian land into West Europe, East Europe, and Asia. During the Early Oligocene (34–32 Ma), the Paratethyan basin began to regress and the Turgai Strait became dry land, which opened up a migration pathway for European and Asian continental animals (11). In the Early Miocene (23–19 Ma), a rapid regression process in the eastern Paratethys enlarged the land areas. The Messinian salinity crisis (5.96–5.33 Ma) was a dramatic drying event of the Mediterranean (8), which might have had a significant impact on endemic aquatic organisms. The subsequent flooding from the Atlantic promoted many surviving species to recolonize the area (13). These geological events and climatic changes in the Tethyan regions are likely associated with the evolutionary history of the genus *Gammarus*.

Current theory predicts that a shift to a new habitat frees species from the competition with closely related species and would increase the rate of diversification followed by adaptive radiations. As lineages diversify into multiple species, competition between newly formed species would intensify. Consequently, the diversification rate would decrease as species accumulate in the limited space (3, 14). In this study, we use DNA sequence data and a variety of analytical methods to test whether the habitat shift of *Gammarus* promoted diversification rates. Phylogenetic inference is used to construct the history of *Gammarus*, to estimate the divergence times of its major lineages, and to determine when the shift from saline to freshwater occurred. We further conduct a biogeographic analysis to explore where *Gammarus* first colonized freshwater habitats and a diversification analysis to assess the temporal diversification mode associated with the habitat shift.

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Results

Phylogenetic Inference Identifies an Eocene Habitat Shift from Saline to Freshwater. We sequenced four genes of 28S, 18S, cytochrome oxidase I (COI), and elongation factor 1 α (EF1 α) from 289 samples representing 115 *Gammarus* species, three Baikal amphipods, and 13 outgroup taxa. Parsimony and likelihood analyses revealed similar topologies, and the likelihood tree is presented in Fig. S1. The genus *Gammarus*, including Baikal amphipods and *Sinogammarus*, which was previously thought to be a junior synonym of *Gammarus* (10), formed a monophyletic group. At the base of the group, there were three saline lineages that are endemic to the Mediterranean–Atlantic area. A freshwater lineage, which is widely distributed in the Palearctic, was nested deeply in the tree. The North American freshwater species formed two groups inside the saline lineages, and they likely had saline common ancestors and colonized freshwater recently. We therefore included them in the saline lineages in subsequent analyses. The freshwater lineage was further divided into four morphologically and geographically distinct clades: the European *Gammarus* clade, which included the *Gammarus fossarum*, *Gammarus pulex*, *Gammarus roeselii*, and *Gammarus komareki* species complexes; the Oriental *Gammarus* clade, which contained all of the Eastern Asian species; the *Gammarus lacustris* lineage and the *Gammarus balcanicus* lineage. These last two clades are primarily located in Europe and Central Asia (Fig. 1).

Divergence time estimates were conducted with a reduced dataset, in which all major clades defined by the phylogenetic analysis were evenly represented to avoid bias. The ages for nodes of interest estimated by BEAST are shown in Fig. 1, and more details are provided in Fig. S2 and Table S2. The genus *Gammarus* originated at ~61 Ma, and started to radiate to freshwater habitat at 43 Ma during the Middle Eocene. The successive freshwater colonizations occurred in the late Eocene, at 36 Ma for the Eurasian *G. lacustris* and *G. balcanicus* clades, at 33 Ma for the European clade, and at 37 Ma for the Oriental clade (Fig. 1). All these freshwater colonization events are older than the earliest *Gammarus* fossil records, which are in the Lower Oligocene.

Biogeographic Analysis Indicates Two Major Range Shifts. We used likelihood and parsimony methods to reconstruct the historical distribution ranges of the hypothetical ancestors. Both methods produced similar results and details are provided in Table S3. Two major range expansion events were detected in the evolutionary history of the genus *Gammarus* (Fig. 1). Ancestral saline *Gammarus* was distributed in the West Palearctic (node b), and subsequently dispersed to the Nearctic at 55 Ma (node j). The second dispersal event occurred within freshwater *Gammarus* (node c), which expanded from the West Palearctic to the East Palearctic at 40.6 Ma (node e).

Diversification Modes Associated with Habitat Shift. We conducted a birth–death analysis to find a model that could best represent the diversification patterns for *Gammarus* overall, freshwater *Gammarus*, and saline *Gammarus*. For *Gammarus* overall, the Monte Carlo constant-rate (MCCR) test rejected the null hypothesis of constant rate (critical value = -3.33 , $P = 0.003$), and the gamma (γ) statistic ($\gamma = -4.3$) indicated a decrease in the diversification rate over time. Of the six models tested, the variable-rate yule2rate model with one shift in diversification was selected as the best fit model for *Gammarus* overall, suggesting that the initial diversification rate of 0.07 species per million years (sp/My) shifted to 0.03 sp/My at 25 Ma. Similarly, the γ statistic and MCCR test significantly rejected the constant-rate model for the freshwater *Gammarus*, and the yule3rate model was chosen as the best fit model with two diversification decreases at 14 Ma (from 0.11 to 0.05 sp/My) and 6 Ma (from 0.05 to 0.01 sp/My). For saline *Gammarus*, the pure birth model with diversification rate of 0.04 sp/My was selected as the best

model, which was supported by the γ and MCCR tests. Detailed results are provided in Table S4.

The semilogarithmic lineage-through-time (LTT) plots for *Gammarus* overall, freshwater, and saline *Gammarus* are shown in Fig. 2 and Fig. S3. The saline *Gammarus* showed a fairly slow but constant rate of lineage accumulation. On the contrary, freshwater *Gammarus* maintained a high diversification rate at the early stages of the colonization of freshwater habitat until ~14 Ma. After that, two independent events of diversification decrease were identified, with 6 Ma being a major shift point.

Absolute net diversification rate within the genus *Gammarus* varied from 0.08 to 0.05 sp/My under two extremes of the relative extinction rate (0 and 0.9). The absolute net diversification rate for freshwater *Gammarus* ranged from 0.10 to 0.06 sp/My, whereas the rates for saline *Gammarus* ranged from 0.05 to 0.03 sp/My. The relative cladogenesis (RC) statistic indicated a significant difference in cladogenesis rate between freshwater and saline *Gammarus* (Fig. 1). Rapid shifts in diversification rate were also located within the freshwater *G. lacustris* and *G. balcanicus* lineages during the Miocene period (Table S5).

Discussion

Saline Origin, Eocene Habitat Shift, and Diversification in Freshwater.

The genus *Gammarus* originated in the West Palearctic with a saline habitat at ~61 Ma in the Paleocene and subsequently colonized a freshwater habitat and radiated across Eurasia starting at ~43 Ma in the Middle Eocene (Figs. 1 and 3, I). During the Paleocene period, the Tethys Ocean stretched out from west to east across Southern Europe and Central Asia, connecting the Atlantic Ocean and the Pacific Ocean (11), where saline *Gammarus* dominated (Fig. 1). When *Gammarus* started to diversify, the exchange between Tethys and Atlantic was free, which may have led to the first range expansion from the Tethys Ocean to both sides of the Atlantic. Along with the progressive crust movements and Tethyan regression during Eocene, the accretion of landmass formed a prominent new freshwater aquatic ecosystem. A euryhaline marine ancestor of *Gammarus* colonized freshwater habitats of the growing Eurasian land, and this habitat shift may have played a significant role in the diversification evolution of *Gammarus*. Our analysis clearly suggests a Tethyan saline origin for *Gammarus*, which contradicts the traditional view that Holarctic freshwater *Gammarus* are of a continental origin and dispersed to coastal seas secondarily (9, 15).

After the successful colonization of freshwater habitats, *Gammarus* underwent a second range expansion from the West Palearctic to the East Palearctic at about 40 Ma and rapidly diverged allopatrically across Eurasian freshwater habitats in the late Eocene. The Oriental *Gammarus* (node i; Figs. 1 and 3, II) separated from its common ancestor with the European freshwater *Gammarus* at 37 Ma, followed by the separation of the Eurasian lineages *G. lacustris* and *G. balcanicus* clade at 36 Ma (node d; Figs. 1 and 3, II). The European freshwater endemic species complexes *G. fossarum*, *G. pulex*, *G. roeselii*, and *G. komareki* diversified at 33 Ma (node h; Figs. 1 and 3, II). Several paleogeological events are likely responsible for these hypothetical radiation events of freshwater *Gammarus*. The collision between the Indian and Asian plates during the Eocene (37–34 Ma) forced the uplift of the Tibetan Plateau, resulting in the interruption of the Tethys Ocean and causing the formation of a freshwater environment in the Eastern Asia. The Turgai Strait, as a shallow seaway, supplied a route for the Paratethyan *Gammarus* to spread northwards and colonize the Eurasian freshwater habitats. The convergence between the African and Eurasian plates during the Oligocene (34–30 Ma) and the subsequent retreat of the Tethys Ocean contributed to the growing European landmass available for *Gammarus* species to inhabit.

Most speciation events that produced the extant species occurred in the Miocene (Fig. 1). The diversification of the

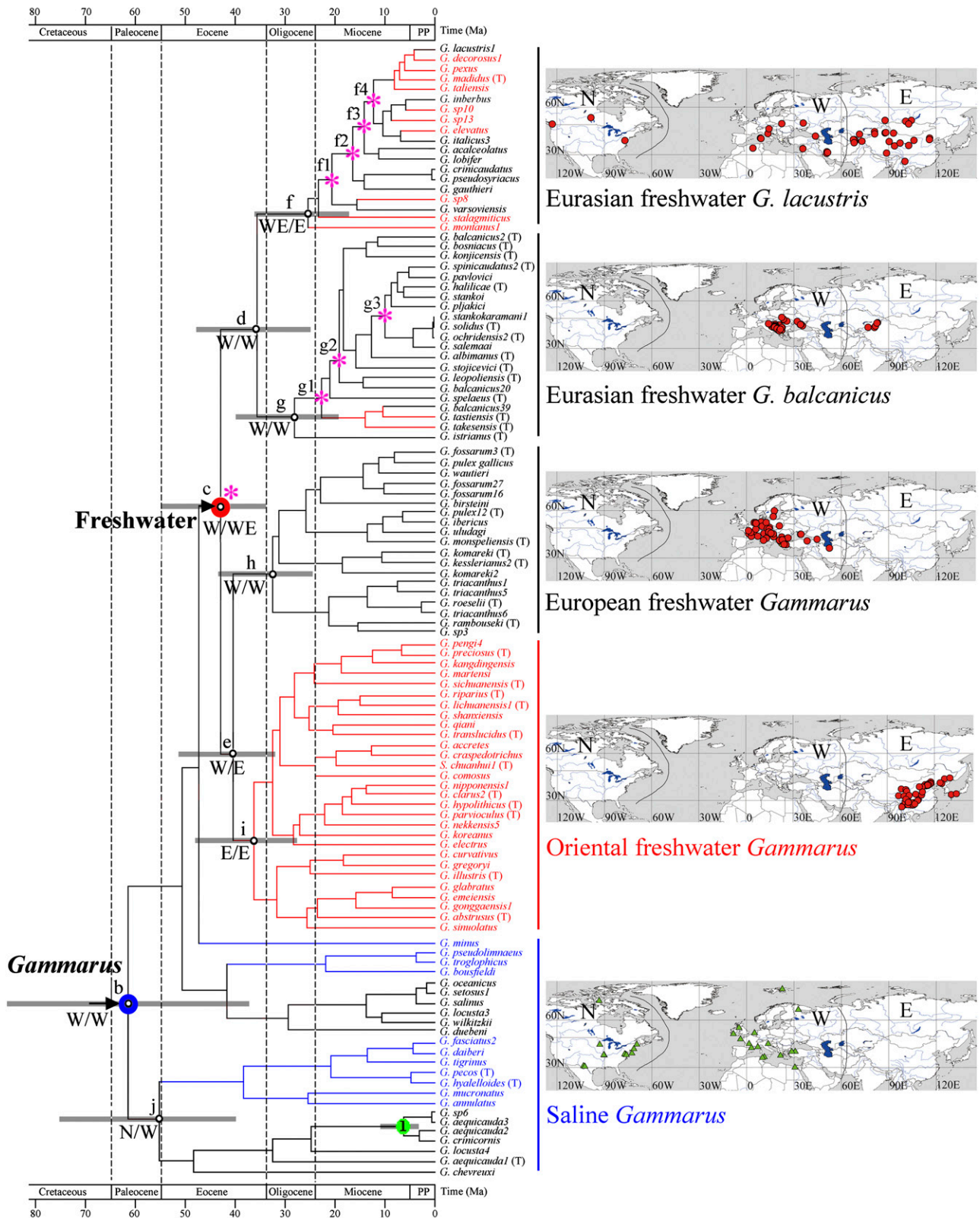


Fig. 1. Maximum clade credibility chronogram inferred from a BEAST dating analysis. Nodes b–j are nodes of interest with gray horizontal bars indicating the 95% highest posterior density interval; node i in green circle is used for calibration. Major ancestral range reconstructions are shown below the branches (N, Nearctic; W, West Palearctic; E, East Palearctic), and arrows show main dispersal events. Species names and terminal branches are in red for East Palearctic species, blue for Nearctic species, and black for West Palearctic species. Pink asterisks are nodes (c, f1–f4, and g1–g3) that showed an increase in diversification rate. The blue circle (node b) indicates the saline origin of the genus *Gammarus* and the red circle (node c) indicates the origin of freshwater species. Outgroups and Baikal amphipods are not shown (Figs. S1 and S2). (Right) Maps correspond to the sampling sites for the adjacent clade.

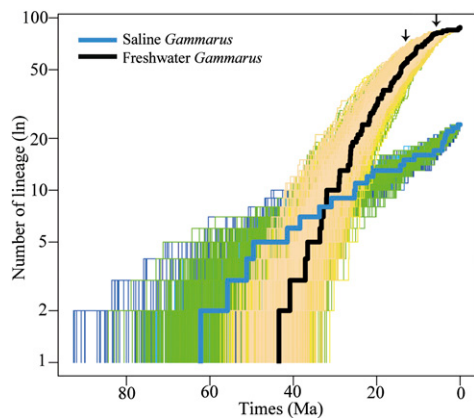


Fig. 2. Lineage-through-time plot estimated from 1,000 Bayesian trees for freshwater and saline *Gammarus*. The lines in bold correspond to the maximum credibility tree from the BEAST dating analysis. Arrows indicate changes in diversification rates that are estimated to have occurred according to the best fit model of lineage diversification.

G. lacustris and *G. balcanicus* clades were probably driven by the closing of the Turgai Strait at ~29 Ma, which made it possible for terrestrial and freshwater animals present in Eurasia to expand their ranges (11). The two lineages were formed at the same time as the closing, and diversified afterward (nodes f and g; Figs. 1 and 3, III). Together, drying of the Turgai Strait, shrinking of the Paratethyan basin, and Eurasian continentalization created available ecological niches for freshwater *Gammarus*. The majority of the freshwater species have a restricted distribution and demonstrate a strong vicariant pattern (9, 10). This is not surprising considering their history and a lack of long-distance dispersal ability. *G. lacustris* is an exception and has a Holarctic distribution. It usually inhabits mountain and glacial lakes and presumably dispersed to northern Europe and North America during glaciations (9).

Habitat Shift Promoted Freshwater *Gammarus* Diversification. Our diversification analyses have revealed that the saline *Gammarus* diversified with a constant rate of 0.04 sp/My throughout its evolutionary history. This is partially expected; as saline *Gammarus* originated from the Tethyan region, they could freely disperse to both sides of the Atlantic without physical barriers. On the other hand, freshwater *Gammarus* initially had a high diversification rate (0.11 sp/My) from the Middle Eocene to Middle Miocene (43–14 Ma), after which the diversification rate decreased to 0.05 sp/My until the Miocene/Pliocene boundary and again declined to 0.01 sp/My toward the modern descendants (Fig. 2 and Table S4). The RC test also suggested that freshwater *Gammarus* went through a rapid diversification within the *G. lacustris* and *G. balcanicus* lineages during the Early and Middle Miocene (Fig. 1). This diversification scenario for freshwater *Gammarus* implies that they experienced a rapid adaptive diversification when they first shifted to a new habitat and that their diversification rate slowed down with the species accumulation, as a model has predicted (3). This finding suggests that freshwater *Gammarus* diversification was driven by ecological opportunities created by the emergence of largely unoccupied habitats and few competitors (2, 16).

The absolute net diversification rate of freshwater *Gammarus* (0.10–0.06 sp/My) is about twice that of saline *Gammarus* (0.05–0.03 sp/My). The rate of freshwater *Gammarus* is comparable with fastest-diversifying bivalves 0.09 sp/My (17) and ground beetles 0.10 sp/My (18). However, it is slower than that of Mediterranean plants, such as *Dianthus* carnations, which have a rate of 2.2–7.6 sp/My (19), and Brassicaceae mustard 0.18–0.22

sp/My (20). The ancient Tethyan region is one of the hotspots of global biodiversity for both terrestrial and aquatic ecosystems. This likely attributes to its complex geological history and heterogeneous topography. This region will continue to provide an excellent setting for studying evolutionary processes.

The genus *Gammarus* is a taxonomically challenging group. Our data unequivocally placed *Sinogammarus* and the Baikal amphipods as part of *Gammarus* (10, 15). The genus “*Sinogammarus*” consists exclusively of cave dwellers, and the Baikal amphipods are endemic to Lake Baikal. Both have highly specialized morphology. We suggest that new synapomorphies should be examined to redefine the genus *Gammarus* and its species groups on the basis of a solid phylogenetic framework.

The hypothesis that ecological opportunities promote lineage diversification is well studied in island terrestrial systems. The genus *Gammarus* presents a strong case in support of this hypothesis in aquatic ecosystems. A habitat shift from saline to freshwater, coupled with increased landmass and thus increased available bodies of freshwater, led to a rapid radiation of freshwater *Gammarus* species. Habitat shift is no rare event in aquatic life, and therefore more studies along these lines are likely to shed more light on the formation of biodiversity.

Materials and Methods

Taxon Sampling. Our sampling covered all major geographic regions across the Northern Hemisphere (Table S1). When possible, samples from the type localities of nominal taxa were included and multiple samples were used to represent widespread species. In total, we used 289 samples of 115 *Gammarus* species inhabiting both freshwater and saline habitats, including two samples of *Sinogammarus*, which is believed to be a junior synonym of *Gammarus* (10). Three Baikal amphipods, which are closely related to *Gammarus* (15), were also included. Another 13 individuals from different genera of the family Gammaridae and several distantly related families were chosen as outgroups. For molecular dating, biogeographic reconstruction, and diversity analysis, unequal taxon sampling could induce bias toward better represented clades (16). Therefore, several taxa were pruned to ensure that all of the major clades recovered in the broader analysis were evenly represented. The reduced matrix consisted of 129 taxa.

Laboratory Protocols and Phylogenetic Analyses. Genomic DNA extraction, amplification, and sequence alignment were performed as in Hou et al. (10). Primers are listed in Table S6. The complete dataset totaled 305 taxa with 5,088 bp, including 1,526 bp for 28S, 656 bp for COI, 2,305 bp for 18S, and 601 bp for EF1 α (Table S1). Nucleotide polymorphisms caused by heterozygosity in EF1 α were limited (less than 10 bp) and were coded as missing data.

Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) criteria. MP analysis and bootstrap support evaluation were conducted using PAUP* 4.0b10 (21). Separated MP analyses of individual genes found no conflict among well-supported nodes (bootstrap index >70%); therefore, the four genes were combined for all other analyses. ML analysis was carried out using GARLI 1.0 (22). To confirm the optimal topology, analyses were repeated until two independently derived topologies of the highest likelihood were identical. A rapid bootstrap analysis with 1,000 replicates was performed under partitioned data mode with RAXML 7.0.3 (23) and a thorough ML search was used to compare with the result of GARLI.

To infer the habitat preference of the hypothetical ancestors, a ML approach was used to map the habitat type onto the *Gammarus* phylogeny (Figs. S1 and S2) with Mesquite 2.74 (24). Freshwater and saline states were coded as binary characters. The proportional likelihoods of ancestral states were calculated for each node under asymmetrical two-parameter Markov k-state model.

Molecular Dating Analysis. Fossil records of amphipod crustaceans are rare and have no detailed affinities with extant taxa (25). Instead, we used three geological events for molecular clock calibration. First, the origin of Mediterranean saline *Gammarus* (node 1, Fig. 1 and Fig. S2) was set at 5 Ma. This is based on the assumption that all gammarids in the region went extinct during the Messinian salinity crisis (8), and that the current lineage was established after the crisis as a consequence of a recolonization of the Mediterranean. Second, the most recent common ancestor of the Baikal clade (node 2, Fig. S2) was set to 30 Ma. The permanent Lake Baikal originated

former were within the confidence intervals calculated by the latter. Calibrations with all three nodes corresponded well with calibration using nodes 1 and 2, whereas calibration using nodes 1 and 3 produced younger estimations. The BEAST conservative estimation with calibration nodes 1 and 2 was therefore reported and used for biogeographic and diversification analyses.

Biogeographic Analysis. To reconstruct the historical distribution range of the hypothetical ancestors, we used a likelihood method implemented in the program Lagrange 20101113 (29). The major areas of *Gammarus* distribution were broadly categorized into three areas: the Nearctic (N), the West Palearctic (W), and the East Palearctic (E) (9). Widespread species were assigned to more than one area (Table S1). The chronogram produced by BEAST was used (Fig. 1), with the outgroups and the Baikal amphipods removed from the tree. The Baikal lineage survived a particularly strong morphological diversification and evolved separately endemic to Lake Baikal (15). Dispersal probabilities were modified to 0.1 between the Nearctic and the East Palearctic or 0.1–1.0 between areas on the basis of paleogeographic reconstructions of area position through time (Table S3) (30).

A parsimony-based analysis was performed for comparison. The same tree topology was used and area assignments with the “maxareas” option unlimited or constrained to two. To estimate the support for ancestral range reconstructions, we randomly sampled 1,000 trees from the BEAST output and conducted a Bayes-DIVA analysis using the program S-DIVA 1.5c (31).

Diversification Pattern. We assigned a conservative estimate of 204 species calculated only for described undisputed species, including 160 freshwater and 44 saline species (32). The diversification pattern was assessed for freshwater, saline *Gammarus* lineages, and *Gammarus* overall chronogram (Fig. 1). To examine the diversification rate change over time, semilogarithmic LTT plots

were constructed in APE 2.5–1 (33). The confidence intervals of the LTT plots were generated on 1,000 trees sampled from the converged BEAST trees.

A birth–death likelihood method was used to test several variable-rate models against the null hypothesis of constant diversification rate with the package LASER 2.3 (34). Akaike information criterion (AIC) scores were computed for the constant-rate and the variable-rate models, including the pure birth, birth–death, yule2rate, yule3rate, variable speciation rate and constant extinction rate (SPVAR), and variable extinction rate and constant speciation rate (EXVAR) models. The AIC difference between best constant-rate model and each of the variable-rate models was computed to select the best fit model. In addition, the MCCR test was used by comparing the empirical γ statistic with the distribution of γ statistics of 1,000 simulated incomplete phylogenies under a pure birth model to test whether the diversification has decelerated through time (35).

The absolute net diversification rates were calculated by using the BEAST chronogram under two extremes of the relative extinction rate (speciation rate/extinction rate = 0 and 0.9) following the whole-clade method (17) implemented in LASER. The RC statistic was calculated with GEIGER 1.3–1 (36) to detect rapid shifts in diversification rates at any specified time. Lineages with more or fewer descendants than expected under the constant-rate model were hypothesized as a diversification rate shift.

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