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Report

Mitogenomic Phylogenetic Analysis Supports Continental-Scale Vicariance in Subterranean Thalassoid Crustaceans

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Summary

Many continental subterranean water crustaceans ("stygobionts") display extreme disjunct distributions, where different species in the same genus are isolated on continents or islands separated by broad oceanic expanses [1]. Despite their freshwater habitat, most of these taxa appear to be most closely related to typical marine groups ("thalassoid" origin) [2]. Among the hadzioids—thalassoid amphipods including the stygobiont families Hadziidae, Pseudoniphargidae, and Metacrangonyctidae—several genera are restricted to inland groundwaters ranging from the Caribbean region to the Mediterranean and Middle East, including interspersed oceanic islands [3]. This distribution might have arisen from Tethyan vicariance [4–7] triggered by the sequential occlusion of the former Tethys Sea, a vast circumtropical ocean existing from the Middle Jurassic up to 20 million years ago (mya). Previous studies have been based on morphological analyses or limited DNA sequence data, making it difficult to test this hypothesis [8–10]. We used complete mitochondrial protein-coding gene sequences, mainly obtained by next-generation sequencing methods and a nuclear ribosomal gene to resolve the phylogeny and to establish a time frame for diversification of the family Metacrangonyctidae (Amphipoda). The results were consistent with the plate tectonics vicariance hypothesis, with major diversifications occurring between 96 and 83 mya.

Results and Discussion

For many years, a key question in zoogeography has been the origin of the extremely disjunct distribution patterns of stygobiont crustaceans after the discovery of Caribbean lineages related to Mediterranean taxa [1]. This distribution pattern exhibited by many genera of thalassoid stygobiont crustaceans is currently explained as a vicariant process whereby plate tectonics caused the fragmentation of a marine ancestor's range, once continuously distributed along the shores of ancient seas [4–6]. This might have been followed by secondary isolation and subsequent speciation in brackish or limnic groundwaters, a process triggered by episodes of sea-level oscillation or of tectonic uplift at coastal areas [5, 7]. Other alternative hypotheses, such as broad-range, open-water marine dispersal are also possible for crustaceans with free-swimming larval stages [1, 11]. In addition, deep-sea dispersal along the crevicular medium associated with the circumglobal system of spreading zones has also been suggested to explain the presence of some of these taxa in geologically young oceanic islands [8, 12].

Testing among these alternative hypotheses requires robust phylogenies and accurate calibration points to derive a reliable estimation of divergence times [8, 9]. Transoceanic dispersal cannot be discarded a priori in stygobiont groups exhibiting a presumed Tethyan distribution but with shallow genetic divergences. Thus, to lend credence to the ancient vicariant origin hypothesis, divergence times between phylogenetic sister lineages placed at opposite sides of the Atlantic should be older than the establishment of deep-water conditions between Iberia and North America at about 95–110 million years ago (mya) [13, 14].

Here we studied the phylogeny of the Metacrangonyctidae, a family of stygobiont amphipod crustaceans with representatives in Hispaniola (Antilles), the Canary Islands, and around the Mediterranean region and the Middle East [15] (Figure 1). This monophyletic taxon [16] comprises two genera, *Metacrangonyx* Chevreux, 1909 and *Longipodacrangonyx* Boutin and Messouli, 1988, including a total of 18 species formally described—11 of which are limited to Morocco—plus at least 18 additional Moroccan taxa still awaiting formal description [16, 17]. The presence of two species of *Metacrangonyx* in the Caribbean region [18] offers an unmatched opportunity to test through phylogenetic analyses the role of dispersal and vicariance in the establishment of disjunct transoceanic distribution patterns in stygobiont crustaceans. We have sequenced the complete mitochondrial genome (~16 kb) (mitogenome) and the nuclear *SSU* ribosomal gene of 21 divergent lineages within 16 metacrangonyctid species and two outgroup taxa (Table 1; see Supplemental Experimental Procedures available online). The data set includes the recently characterized mitogenome of *Metacrangonyx longipes* Chevreux, 1909, from Mallorca (Balearic Islands) [19]. Species were chosen to cover the major genetic and geographic lineages revealed in a preliminary mitochondrial phylogenetic analysis based on cytochrome oxidase subunit 1 (COI) and 16S RNA (*rrn*L) gene fragments (Figure S1; Table S1). Most of the mitogenomes considered herein were obtained with 454 sequencing technology, using multiplexing by parallel-tagged libraries or by pooling untagged amplicons [20, 21]. Our main aims were (1) to derive a strongly supported phylogeny of the Metacrangonyctidae, sampling their full geographic distribution and (2) to estimate tree node ages using molecular dating techniques and paleogeographic calibration points. We aimed to test the hypothesis that species at opposite sides of the Atlantic had a vicariant origin, not a Mediterranean source followed by a secondary dispersal to the Caribbean.

Mitogenomic Phylogenies Solve Evolutionary Relationships within the Metacrangonyctidae

A Bayesian tree (Figure S2) was built using the full set of mitochondrial protein-coding genes (MPCGs) by implementing the

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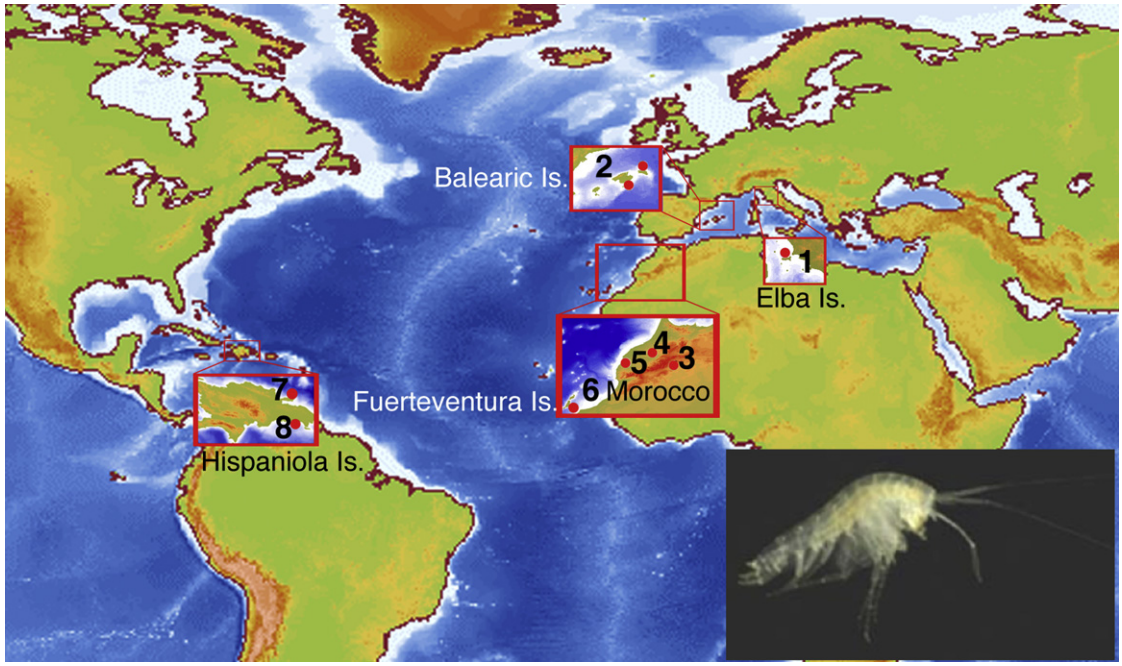

Figure 1. Metacrangonyctid Species Distribution

Map showing approximate geographic sampling locations of metacrangonyctid species for which whole mitochondrial genomes have been sequenced. See Table 1 and Table S1 for precise distribution of each taxon. 1, *Metacrangonyx ilvanus*; 2, *M. longipes*; 3, "M. notenboomi," *M. goulmimensis*, *M. longicaudus*, *M. spinicaudatus*, "M. paurosexualis," *M. panousei*; 4, *M. remyi*, "M. boveei"; 5, "L. stocki," "M. boutini boutini," "M. nicoleae tamri"; 6, *M. repens*; 7, *M. samanensis*; and 8, *M. dominicanus*. Inset shows *M. dominicanus* (photo by T.M. Iliffe). See also Figure S1.

best partition scheme and evolutionary models selected using the Bayesian Information Criterion (BIC; see Supplemental Experimental Procedures). Similar tree topologies were obtained using other partition schemes or the protein data set, but with discrepancies in the relationship among the taxa from Atlantic Morocco (they appeared as monophyletic in some analyses but paraphyletic in others) and in nodes relating the five insular species (Figure S2). The tree topology based on *SSU* sequences showed congruence with that obtained with MPCGs, but posterior probability values were low, particularly for basal nodes (Figure S2). The combined mitochondrial + *SSU* analyses supported paraphyly of the Atlantic Morocco lineages (Figure 2; Figure S2), but a Shimodaira-Hasegawa test showed that alternative topologies were not significantly different.

The tree recovered in the combined analysis (Figure 2) that we regard as our best phylogenetic hypothesis shows five strongly supported major clades within the family Metacrangonyctidae (here named A, B, C, D1, and D2). Each of these has a clearly delimited geographic projection, although B and C share the same overall area in Morocco. All tree topologies agreed on the assignment of an early divergence to "M. boveei" (tentative binomen) and *M. remyi* (clade A), which occur in the northern valleys and springs of the Western High Atlas in Morocco (Figures 1 and 2). Clade D appeared further subdivided into two strongly supported subclades: D1, present at both sides of the High Atlas in Morocco; and D2, embracing the five insular *Metacrangonyx* species from Mallorca-Menorca and Elba in the Mediterranean, Fuerteventura in the Canary Islands, and Hispaniola in the Caribbean. A Partition Bremer Support test taking each of the 13 MPCGs as a different partition suggested that the lack of resolution within the island subclade D2 arose from an absence of phylogenetic signal and not from incongruence among gene partitions (Figure S2). A further Bayesian analysis implementing a polytomy prior in Phycas (see Supplemental Experimental Procedures) also led to the rejection of a fully resolved tree in favor of a hard polytomy.

Several recent attempts to resolve the phylogeny and to explain the disjunct distributions of some atyid shrimp [9], remipedes [8], and cirolanid isopods [10] have been based only on partial mitochondrial and nuclear DNA sequences. Although mitogenome-based phylogenies represent a single locus and do not necessarily reflect the correct species tree, they have a considerably higher resolution power than partial (mitochondrial or nuclear) DNA sequences because of the large number of nucleotide positions considered and the high mutation rate exhibited by MPCGs. Our analysis of the phylogenetic relationships within the Metacrangonyctidae based on the 13 MPCGs with a partitioning by codon position plus the *SSU* nuclear marker produced an almost fully resolved tree except for the hard polytomy affecting insular clade D2.

Time Frame for Metacrangonyctid Diversification

Following the rejection of a strict molecular clock, we estimated node ages enforcing a relaxed molecular clock on the combined analysis topology assuming three independent substitution rates for each mitochondrial codon position. Two paleogeographic events were used to calibrate the tree. The divergence of the two lineages of *M. longipes* present on the Balearic archipelago was assumed to be associated with the complex geologic events that occurred in the Western Mediterranean from the Middle to the Late Miocene. Namely, the age for the complete separation of the Balearic Islands from other continental microplates detached from the Iberian

Table 1. Collection Sites and Mitochondrial Genome Sequence Information for Species Included in Analyses

Species	Locality	Mitogenome Length (bp)	*SSU* Length (bp)	Average Read Length (bp)	Coverage	Acc. N. Mitogenome	Acc. N. *SSU*
M. dominicanus Jaume and Christenson, 2001	Juan Dolio, S. Hispaniola (Dominican Rep.); well	14,543[d]	2,511	625	4.3×[a]	HE860499	HE967299
M. samanensis Jaume and Christenson, 2001	Samaná, N. Hispaniola (Dominican Rep.); well	14,067[f]	2,413	551	5.7×[a]	HE860505	HE967297
M. repens (Stock and Rondé-Broekhuizen, 1986)	Fuerteventura, Canary Is. (Spain); well	14,355	2,215	550	5.7×[a]	HE860495	HE967284
"M. nicolae tamri"	Aksri, NW Agadir (Morocco); spring near Talmat cave	13,517[e]	1,027+1268	411	145x[c]	HE860511	HE967292-3
"M. nicolae tamri"	Tamri, N. Agadir (Morocco); well	14,644	2,415	595	59×[b]	HE860504	HE967294
"M. boutini boutini"	Timzelite, Souss Massa NP, S. Agadir (Morocco); well	13,301[e]	2,357	433	107×[c]	HE860497	HE967295
M. panousei Balazuc and Ruffo, 1953	Agdz (Morocco); well	14,478[d]	2,295	453	80×[c]	HE860510	HE967289
M. goulmimensis Messouli, Boutin, and Coineau, 1991	Lamkedmyia Meleh Jorf, NW Erfoud (Morocco); well	14,507[d]	1,173	413	178×[c]	HE860500	HE967279
M. goulmimensis Messouli, Boutin, and Coineau, 1991	Ousroutou, E Rich, N. Errachidia (Morocco); well	14,602	2,179	454	100×[c]	HE860501	HE967278
M. goulmimensis Messouli, Boutin, and Coineau, 1991	Zouala maisson, S. Errachidia (Morocco); well	14,353	1,922	423	165×[c]	HE860502	HE967280
M. longicaudus Ruffo, 1954	Lamkedmyia Meleh Jorf, NW Erfoud (Morocco); well	14,712[d]	2,272	403	135×[c]	HE860509	HE967281
"M. notenboomi"	Maadid, NE Erfoud (Morocco); well	14,277[d]	2,275	420	168×[c]	HE860513	HE967298
"M. paurosexualis"	Souk Tben, Haouz Plain, Marrakech (Morocco); well	12,542[e]	2,370	424	94×[c]	HE860507	HE967291
M. spinicaudatus Karaman and Pesce, 1980	Souk Tben, Haouz Plain, Marrakech (Morocco); well	15,037	2,338	534	124×[b]	HE860506	HE967290
M. remyi Ruffo, 1953	Ijoukak, Western High Atlas (Morocco); spring at maison forestière	14,787[d]	2,246	454	281×[b]	HE860512	HE967287
"M. boveei"	L'Ourika valley, Western High Atlas (Morocco); well	15,012	2,299	511	100×[b]	HE860498	HE967288
"Longipodacrangonyx stocki'	Tafraut (Morocco); well	12,924[e]	337+1210	395	160×[c]	HE860496	HE967282-3
"Longipodacrangonyx stocki"	Arbaa-Sahe, SW Tiznit (Morocco); well	13,006[f]	N/A	431	103×[c]	HE860508	N/A
M. longipes Chevreux, 1909	Mallorca, Balearic Is. (Spain); Cala Varques Cave	14,113	2,200	NA	NA	AM944817	HE967285
M. longipes Chevreux, 1909	Menorca, Balearic Is. (Spain); Cala Figuera cave	14,117	2,087	437	87×[c]	HE861923	HE967286
M. ilvanus Stoch, 1997	Elba Island (Italy); well	14,770[d]	1,173	540	78×[b]	HE860503	HE967296
Pseudoniphargus daviui Jaume 1991	Cabrera, Balearic Is. (Spain); well	15,155	1,863	410	73×[b]	FR872383	HE967300
Bahadzia jaraguensis Jaume and Wagner 1988	Oviedo; S. Hispaniola (Dominican Rep.); cave	14,657	N/A	537	87×[b]	FR872382	N/A

See Table S1 for precise locations. Species names in quotes and not in italics denote taxa not formally described yet [17].
[a]Sanger method.
[b]Roche FLX/454 with tagging.
[c]Roche GS Junior with no tagging.
[d]Partial mitogenome due to A-T rich region was not completely sequenced.
[e]Partial mitogenome due to fragment comprising *rrn*L-*trn*S region was not completely sequenced.
[f]Partial mitogenome due to fragment including *rrn*S gene and A-T rich region was not completely sequenced.

rim (16 mya) and the Messinian Salinity Crisis (5.5 mya) were taken as the upper and lower bounds, respectively ([22, 23] and references therein). Furthermore, we assumed that the age of the node corresponding to the sister species *M. remyi* and "M. boveei," which dwell in neighboring valleys of the northern slopes of the Marrakech High Atlas in Morocco, cannot be younger than the uplift of this mountain range. According to [24], the Atlas domain remained submerged until the Middle Eocene (48.6–37.2 mya), the earliest uplift being dated as Late Eocene (37.2–33.9 mya), whereas the first significant folding extended through the entire Oligocene until the Early Miocene (25 mya). Thus, we propose to assign an age interval of 37.2–25.0 million years (my) to the last common ancestor of the two species.

Our chronogram (Figure 3) assigned an age of 96 my (with 95% higher posterior densities [HPDs] of 71–125 my) to the initial diversification that led to the contemporary metacrangonyctid lineages and suggests a mid-Cretaceous origin for the main clades of the family. Cross-validation showed that node ages estimated assuming the two foregoing paleogeographic events separately or by implementing different diversification models fell within the 95% HPDs of the estimations derived after considering the two calibration points altogether (Figure S3). An old lineage diversification at a remarkably small geographic scale took place in Morocco, where four of the five recognized monophyletic lineages concur, one of them resulting to be sister to the insular clade. Our estimated ages agree in general terms with those of Boutin [25] in the

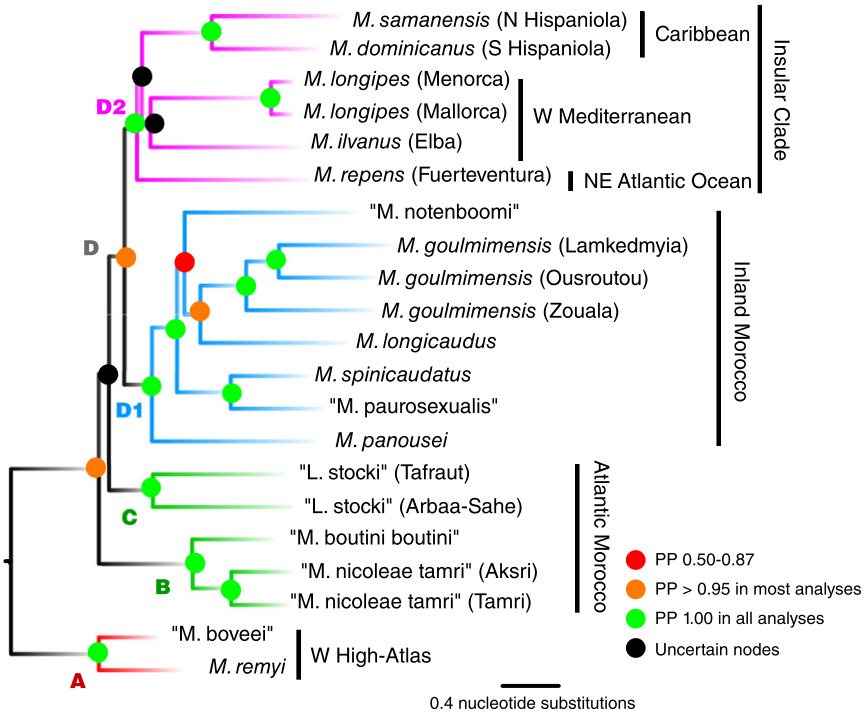

Figure 2. Mitogenomic + *SSU* Phylogenetic Tree of the Family Metacrangonyctidae

The topology was obtained from Bayesian analysis of combined mitochondrial protein-coding genes and 18S rRNA (*SSU*). The best partitioning scheme was selected based on Bayesian Information Criterion (GTR+I + Γ in first/second positions as different partitions, HKY+I + Γ for third mitochondrial codon positions and GTR+I + Γ for *SSU*) (see Table S2). Monophyly of the Metacrangonyctidae was supported with maximum posterior probability in all analyses (outgroups not shown). Dots of different colors at nodes summarize posterior probability support values obtained using different methods (details on topologies and support values for each analysis are shown in Figure S2).

assignment of a remarkably old age to the Metacrangonyctidae, with the differentiation of the major lineages in our phylogenetic reconstruction corresponding to the Cretaceous, c. 96–83 mya. This age range is coincident with the major Late Cretaceous Cenomano–Turonian transgression–regression cycle that affected most of the current Moroccan geography, and it was suggested to be the cause of individualization in continental groundwaters of some Moroccan lineages [25]. Our phylogeny shows that the insular species of *Metacrangonyx* (including the Caribbean and Mediterranean taxa) formed a strongly supported monophyletic group in all analyses (subclade D2), despite their extremely disjunct distribution. Remarkably, *M. repens* from Fuerteventura (Canary Islands) belongs to this insular clade and not to any of the geographically closer Moroccan clades. Two unresolved nodes account for the relationship between the Mediterranean insular species (Elba and Balearic Islands) and those of the Atlantic (Fuerteventura and Hispaniola). It has been suggested elsewhere that the difficulty in resolving phylogenetic relationships in subterranean amphipods might arise from the occurrence of sudden radiations associated with the synchronous colonization of groundwater by different populations of the same ancestor [2,26]. Our finding that the insular clade of *Metacrangonyx* diversified basally as a true polytomy is in agreement with the hypothesis of a simultaneous divergence ultimately leading to speciation in the isolated populations. The initial diversification of the insular clade is estimated to have occurred at 79 mya (95% HPDs 60–108 mya), a time frame compatible with the plate tectonics vicariance hypothesis if we consider the uncertainties associated with both the tectonic reconstruction of Tethys history and molecular clock estimations. Our estimated age for the divergence between the metacrangonyctids of Hispaniola and their Balearic sister group (77 mya; 95% HPDs 57–101 mya) lends additional support to this hypothesis. During that epoch, the Caribbean, the East Atlantic, and the portion of the Tethys Sea placed at the position of the Western Mediterranean formed a continuous seaway, and their shores and islands were placed much closer to each other than at present [27]. Note that Hispaniola Island, the Canary Islands and the entire Western Mediterranean basin had not yet formed at that time. The current Caribbean configuration is no older than the Late Eocene in age [28]; Fuerteventura, the oldest island of the Canary archipelago, dates back to 22 mya [29], whereas the entire Western Mediterranean basin formed at c. 20 mya [30]. However, there is compelling geologic evidence for the presence of drowned archipelagos and seamounts in the central East Atlantic since at least 60 mya [31–33]. These Paleo-Macaronesian islands were located much closer to the Western Mediterranean than today [33]. Ephemeral islands likely lasting a few million years each have been present in the Proto-Caribbean (volcanic islands, shallow banks and ridges) since the early Cretaceous [28]. Thus, the existence of these vanished archipelagos makes it likely that the ancestor of the insular lineage of *Metacrangonyx* was a shallow-water marine species that populated islands, shallow banks or strips of coast placed in this overall area, but not in the precise locations occupied by modern species.

Across the Metacrangonyctid mitochondrial protein-coding genes, we found an average long-term evolutionary rate of pairwise sequence divergence of 10.9% per million years. This is almost five times higher than the "standard" 2.3% of arthropod mitogenomes [34] and beetle MPCGs [35], or other rates estimated for the COI gene in marine decapods (1.4%–2.6% per million years) that have been applied frequently to other crustaceans [36]. If the "standard" 2.3% rate is used the estimated ages would be even older, thus not contradicting the main conclusion of an ancient vicariance. The only similar estimate to our knowledge is the 20% COI rate per million years obtained in the Hawaiian stygobiont decapod *Halocaridina rubra*, an accelerated evolution that has been related to the strong genetic structure and to the frequent occurrence of population bottlenecks in this species [37].

Other stygobiont crustacean groups such as remipedes and some ostracod, copepod, thermosbaenacean, and decapod lineages also display a presumed Tethyan distribution [38]. A clarification of their molecular phylogenies and timing of cladogenesis could shed light on the origin of their distribution

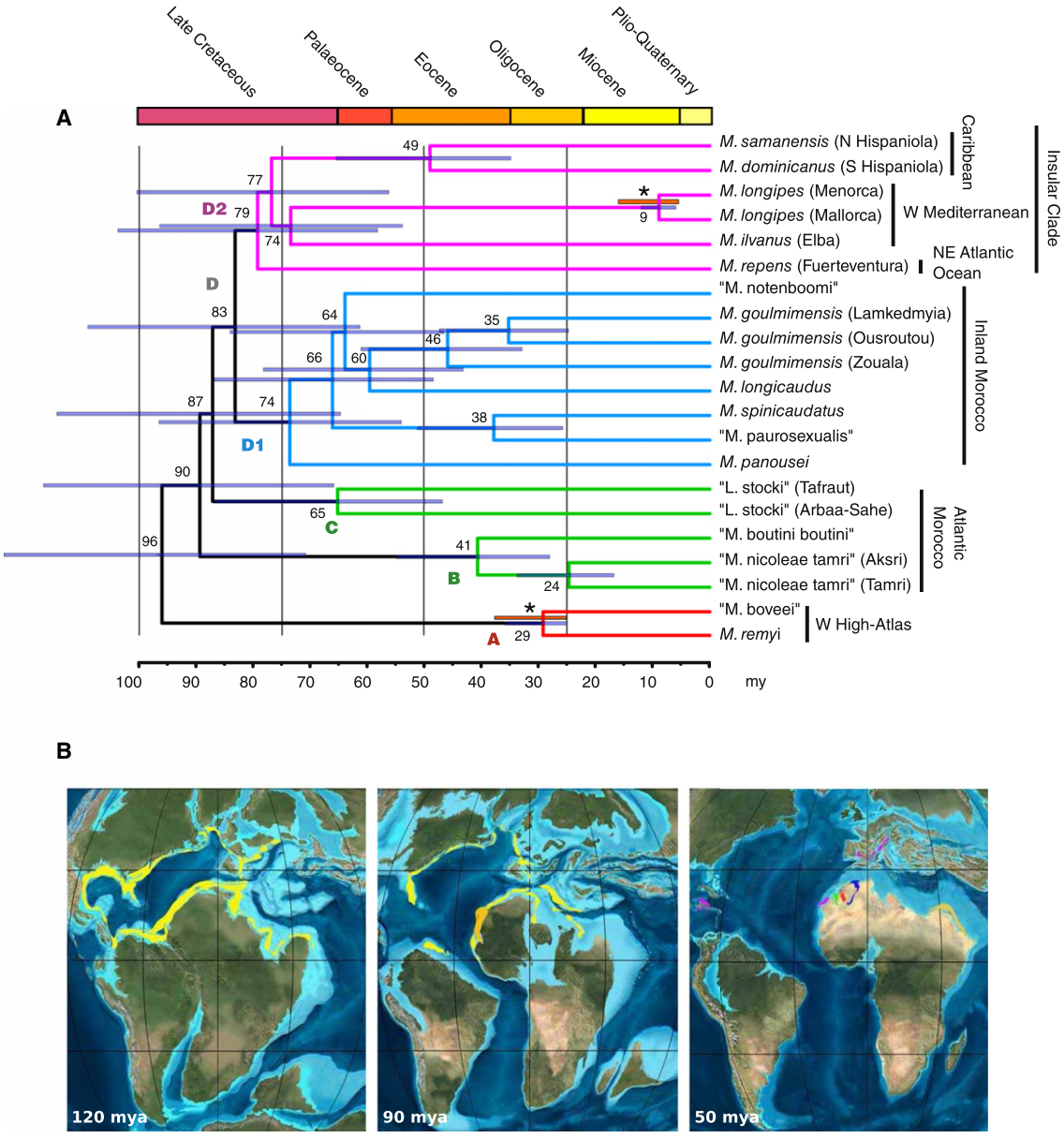

Figure 3. Time Frame for Diversification of the Metacrangonyctidae

(A) Shown are the divergence times for the Metacrangonyctidae estimated from Bayesian analysis of all mitochondrial protein-coding gene sequences based on two paleogeographic calibration points and a Yule diversification model (see main text and Supplemental Experimental Procedures). Mean values are indicated on nodes, whereas horizontal bars across nodes represent the 95% highest probability density intervals. Asterisks identify node constraints, with their respective age ranges in red, implemented as flat priors in the analysis. See also Figure S3 for ages estimated using single calibration points, strict clock, and Birth-Death models.

(B) Maps show global paleogeography at three different periods with corresponding putative metacrangonyctid distributions. Maps modified from Ron Blakey, NAU Geology (http://jan.ucc.nau.edu/~rcb7/globaltext2.html).

patterns in the context of the fragmentation of the Tethys Sea. Extensive mitochondrial data sets in combination with multiple nuclear loci obtained by next-generation DNA sequencing are a promising source of genetic information to unravel the process and timing of diversification of these stygobiont crustaceans.

Accession Numbers

Sequences obtained for this paper were deposited under EMBL accession numbers HE967026–HE967186 for COI, HE967187–HE967277 and HE970657–HE970663 for *rrn*L, FR872382-FR872383, HE860495-HE860513, and HE861923 for mitogenomes, and HE967278-HE967300 for *SSU*.

Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.09.012.

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