

Phylogenetic relationships of sleeper gobies (Eleotridae: Gobiiformes: Gobioidae), with comments on the position of the miniature genus *Microphilypnus*

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Abstract

Microphilypnus and *Leptophilypnion* are miniaturized genera within the family Eleotridae. The evolutionary relationships among these taxa are still poorly understood, and molecular analyses are restricted to mitochondrial genes, which have not been conclusive. We compiled both mitochondrial and nuclear genes in order to study the phylogenetic position of *Microphilypnus* and the evolutionary history and relationships of eleotrids. We propose that *Microphilypnus* and *Leptophilypnus* (a non-miniature genus) are not sister groups as suggested by previous studies, but rather separate lineages that arose in the early Eocene, with *Leptophilypnus* recovered as a sister group to the other analyzed eleotrids. In fact, *Microphilypnus* is currently associated with the Neotropical clade *Guavina/Dormitator/Gobiomorus*. We also identified a well-supported clade that indicated *Gobiomorus* and *Hemieleotris* as paraphyletic groups, besides a close relationship among *Calumia godeffroyi*, *Bunaka gyrinoides*, *Eleotris* and *Erotelis* species. This is the first comprehensive report about the evolutionary relationships in members of the family Eleotridae, including multiloci and multispecies approaches. Therefore, we provided new insights about the phylogenetic position of some taxa absent in previous studies, such as the miniature genus *Microphilypnus* and a recently described species of *Eleotris* from South America.

Introduction

The family Eleotridae (Bonaparte, 1835), whose members are popularly known as “sleepers” and “gudgeons”, is the second most diverse fish family of the order Gobiiformes, comprising 142 species from 35 genera widespread in tropical and subtropical waters in the Neotropics, Africa, and the Indo-Pacific^{1,2,3}. Most eleotrids inhabit brackish or freshwater environments, albeit a few species are truly marine⁴. Furthermore, some freshwater species have a marine larval stage returning to freshwater as juveniles⁵. They are carnivores that feed on crustaceans and other benthic invertebrates, small fishes, and insects, although the marine larval stages of some species feed on plankton^{6,7}.

Molecular phylogenetic studies have provided significant insights into the evolutionary history of Eleotridae in the past decades, even though some phylogenetic relationships in this group remain uncertain. For example, Thacker & Hardman⁴ suggested that the clade composed of *Microphilypnus/Philypnodon/Leptophilypnus* would be the sister-group of all other eleotrids, including the species from Oceania and the Indo-Pacific region. Nonetheless, Thacker⁸ noted that the clade *Microphilypnus/Leptophilypnus* appears to be the sister-group to the Neotropical clade (*Dormitator/Guavina/Gobiomorus*), while *Philypnodon* is more closely related to the genus *Gobiomorphus* from Oceania (Fig. 1).

The phylogenetic position of the Indo-Pacific species *Calumia godeffroyi* is also controversial. Thacker et al.⁴ proposed a clade formed by *Calumia* and *Hypseleotris*, while Thacker (2009) suggested a relationship between *Calumia* and *Eleotris*, whose species have a circumtropical distribution.

Subsequently, Chakrabarty et al.⁹ recovered *Calumia* as a sister group of the genus *Gobiomorphus*, native to New Zealand and Australia (Fig. 1).

Although these studies have shed light on general aspects of evolutionary relationships within the family, the genealogies were based solely on mitochondrial DNA (mtDNA). Despite the recurrent use of mitochondrial sequences to solve long-standing phylogenetic problems over the past few decades, phylogenies based on single genes (considering that mtDNA behaves as a single genetic locus) provide limited phylogenetic signals and therefore single-locus inferences might be biased^{10,11}. In this sense, combining nuclear (nuDNA) and mitochondrial DNA (mtDNA) genes can provide finer descriptions of phylogenetic and phylogeographic scenarios, being more efficient than studies based only on mtDNA markers^{12,13,14,15,16,17,18}. Furthermore, it is now widely accepted that combining information from multiple loci using methods that account for stochastic processes during evolution improves the inferences about the historical diversification of organisms^{19,20,21}.

Miniaturization in eleotrids. Miniaturization is an evolutionary process that leads to reduced body size of lineages over time, being observed in several groups of fishes, amphibians, reptiles, and primates^{22,-24}. In general, miniaturization is accompanied by structural simplifications, novel structures, and increased variation²⁵. In some cases, the truncated development eventually determines the appearance of distinct evolutionary novelties, including “bizarre” forms^{26,27}.

The miniature taxa (traditionally defined as those species with a total length below 25 mm) are particularly diversified in ichthyofauna²⁸. For example, only in the Neotropical region, nearly 210 miniature fish taxa have been reported encompassing the main orders: Characiformes, Siluriformes, Cyprinodontiformes, Perciformes, and Gobiiformes²⁹. In the order Gobiiformes, two miniature genera of the family Eleotridae are recognized, namely: *Microphilypnus*³⁰⁻³³, and *Leptophilypnion*³⁴.

Although the description of miniature forms and their adaptive relationships have been addressed since classical works by Haeckel.³⁵, current phylogenetic approaches might elucidate the tempo and mode of evolution in body size of species and/or lineages^{36,37}. However, the few studies available based on molecular data involving the miniature genera *Microphilypnus* have generated contrasting evolutionary scenarios. ^{4,8} hypothesized a phylogenetic relationship between miniaturized species of the genus *Microphilypnus* with non-miniaturized genus from coastal rivers in Central America *Leptophilypnus*. On the other hand, Thacker³⁸ demonstrated that *Microphilypnus* is more closely related to another non-miniature genus (*Philypnodon*), endemic to freshwater ecosystems in Australia. Therefore, further investigations are required to provide a reliable phylogenetic reconstruction about the evolutionary transition from non-miniature to miniature groups, and thus, determine whether miniaturized genera represent a miniaturized clade or the result of independent evolutionary events of miniaturization.

Therefore, to refine the evolutionary relationships within Eleotridae, we generated a more comprehensive phylogeny of this family based on mitochondrial and nuclear genes, including taxa excluded from

previous phylogenetic reports and comprising different biogeographic regions. Although molecular data from representatives of the miniature genus *Leptophilypnus* are still absent, our multi-locus phylogenetic analyses allow exploring the phylogenetic position of the miniaturized group *Microphilypnus*, which has often been neglected in former studies.

Results

The final dataset in molecular phylogenetic analyses consisted of 52 taxa, being four of them related to outgroups. The final concatenated alignment of mitochondrial and nuclear DNA sequences had a length of 3,494 bp (16S, COI, ND2, Rhod, EGR1) and the phylogenetic reconstruction was based on the Bayesian coalescence approach (species tree on *BEAST) (Fig. 2). The two exons used had a total of 247 variable sites in 1240 bp (EGR1 = 118/811, and Rhod = 129/429). The dataset partitioning scheme and the nucleotide substitution models for multilocus phylogenetic analyses are shown in Supplementary Table S2.

The phylogenetic reconstruction confirmed the monophyly of the family Eleotridae and revealed six well-supported main clades (> 0.95 PP): 1) *Eleotris* / *Erotelis* / *Bunaka* / *Calumia*; 2) the Australian genus *Gobiomorphus* / *Philypnodon*; 3) the Neotropical genus *Dormitator* / *Guavina* / *Gobiomorus* / *Hemieleotris* / *Microphilypnus*; 4) the Australian genus *Giuris* / *Mogurnda* / *Ratsirakia* / *Tateurdina*; 5) genus *Hypseleotris*; and 6) genus *Leptophilypnus* (Fig. 2).

The first clade (in blue) includes *Eleotris* from Neotropical and Indo-Pacific regions. The relationships between *Bunaka gyrinoides* and *Calumia godeffroyi* and the species of the genus *Eleotris* and *Erotelis* were strongly supported (PP > 0.99). We found that *Eleotris* species from the western Atlantic (*E. amblyopsis*, *E. pisonis*, *E. perniger*, and the recently discovered lineages but not formerly described (*Eleotris* sp. 1 and *Eleotris* sp. 2) form a monophyletic group. Phylogenetic analyses showed that the neotropical species are not monophyletic (blue, green, and red clades). Instead, the clade from Australia and New Zealand (yellow clade) is a sister group of the *Eleotris* lineage (PP = 0.99). In this clade, *Gobiomorphus* (represented by species from Eastern Australia and New Zealand) was recovered as a sister group of *Philypnodon* (Eastern Australia). Also, the close relationship between *Guavina* and *Dormitator* (Neotropical region) was well supported (PP = 1.0), while *Dormitator latifrons* (from Western Pacific) has been recovered as a sister lineage in relation to *D. maculatus* (Western Atlantic), *D. cubanus* (Cuba) and *D. lebretonis* (Western Africa).

The miniature fish of the genus *Microphilypnus* were placed in a phylogenetic framework with eleotrids species from different biogeographic regions, suggesting a close evolutionary relationship with high support values (PP > 0.98) between this group (represented by *M. ternetzi*, *Microphilypnus* sp. 1. and *Microphilypnus* sp. 2) and the neotropical clade, comprising *Guavina*, *Gobiomorus* and *Hemieleotris* (non-miniature genera). Molecular phylogeny supports the paraphyletic nature of the genus *Gobiomorus*, since *Hemieleotris latifasciata* appears nested in the *G. dormitor* / *G. polylepis* / *G. maculatus* clade. Finally, our phylogenetic analyses recovered the dichotomy of the Neotropical freshwater species

Leptophilypnus fluviatilis and *L. panamensis* as a sister group of all eleotrid species herein analyzed (PP = 1.0).

Estimates of divergence time in Eleotridae. We estimated the origin of the clade Eleotridae back to Early Eocene (55.6 Ma, IC = 53.6–57.5 95% highest posterior density - HPD), which corresponds to the split between the ancestral lineage of *Leptophilypnus* and the remaining eleotrids, with the subsequent diversification events occurred during the transition from Oligocene to Pleistocene. The clade including the miniature *Microphilypnus* species and the neotropical lineages (*Dormitator* / *Guavina*) has diverged during the Miocene (mean estimated date 16.6 Ma, 95% posterior credibility interval = 14.7–18.5 Ma). The most recent divergence events have taken place in Pleistocene (1.4 Ma) between *Dormitator cubanus* (from Cuba) and *D. lebretonis* (from Eastern Central Atlantic), followed by the the split between *Eleotris acanthopoma* (Southeastern Asia) and *E. sandwicensis* (Hawaiian Islands) (1.6 Ma) (Fig. 2). The divergence among the lineages from the intercontinental clade composed of *Philypnodon* (Eastern Australia) and *Gobiomorphus* (Eastern Australia and New Zealand) appeared to have occurred during the Oligocene (27.7 My 95% IC 21.0 – 34.2 My).

Discussion

The present study is the first to provide a phylogenetic reconstruction of the family Eleotridae based on multiple loci (mtDNA and nuDNA). Based on these results, we inferred phylogenetic hypotheses to shed light on the evolutionary history of freshwater and estuarine eleotrids, encompassing the evolutionary relationships among 48 species.

Miniaturization is a recurrent theme in evolutionary studies since this phenomenon involves processes related to the reduction of body size usually associated with remarkable changes in morphology, physiology, ecology, life history, behavior, and reproductive maturity of organisms²⁵. From a genetic point of view, phylogenetic approaches can greatly contribute to unraveling the evolution of miniature species. For example, the phylogenetic position of the miniature genus *Paedocypris*, considered one of the smallest groups of vertebrates (standard length of 10–12 mm), was determined based on inferences from mitochondrial DNA (cytochrome b)³⁶, who located *Paedocypris* as a sister group to the miniature species of the genus *Sundadanio*, both of which were found to be sister lineages and the other taxon within the family Cyprinidae. Later,⁵¹ provided a more consistent phylogenetic signal of this group based on six nuclear genes, where *Paedocypris* appears as a sister group to all cyprinids. Both reports indicated that the miniaturization processes have taken place independently.

In the case of Eleotridae, previous phylogenetic reconstructions based on mitochondrial genes corroborated *Microphilypnus* and the non-miniature genus *Leptophilypnus* as sister groups^{4,8,9,52} (Fig. 1). Here, we found strong support in the species tree that included the miniature species of *Microphilypnus* within the Neotropical clade *Dormitator* / *Guavina* / *Gobiomorus* / *Hemieleotris* (Fig. 2). Although we have no representatives of the miniaturized genus *Leptophilypnion* in our phylogeny, the miniaturization in Eleotridae has probably arisen, at least, in two moments during their evolution.

Even though the polyphyletic status of *Microphilypnus* and *Leptophilypnus* remains unknown, there are no synapomorphies described for both clades so far. *Microphilypnus* presents a suite of morphological features not found in *Leptophilypnus* and most other eleotrids such as reduction of pectoral-fin rays (11-15- vs. 15 or more), lateral ethmoid barely ossified and slender in frontal view (vs. ossification and cone-shaped in frontal view), and not ossified scapula in adults (vs. ossified). *Leptophilypnus* shares with *Microphilypnus* some reductive features like the slender infraorbital region and the lack of a series of infraorbital papillae absent. Nonetheless, features are likely to have evolved repeatedly in both lineages as the result of independent miniaturization events.

It is noteworthy that miniaturization events are often reported in Gobiiformes, suggesting a trend in this group towards the reduction of body size and loss of some morphological traits associated with miniature forms. Besides, the parallel adaptive evolution to similar microhabitats eventually leads to homoplasy, thus hindering the establishment of reliable phylogenetic relationships based only on morphology. On the other hand, the emergence of miniature and phylogenetically divergent groups supports our hypothesis that the miniaturization processes in Eleotridae represent independent evolutionary pathways.

Unfortunately, the phylogenetic position of *Leptophilypnion*, a recently described genus of Neotropical miniature eleotrids³⁴, remains obscure. According to morphological traits, *Leptophilypnion* would be more related to *Microphilypnus* than to *Leptophilypnus*, by sharing some features such as the reduction in the number of scales and pectoral fins. Nonetheless, *Leptophilypnion* is distinguished by the presence of elongated pelvic fin rays, five branchiostegal rays (vs. six in other eleotrids), and additional unusual characters in skeleton³⁴. Therefore, further information is needed to resolve the evolutionary relationships between species of these groups.

Eleotris corresponds to the only genus of Eleotridae that is widely distributed in different biogeographic areas, from the Neotropics, Africa, Indo-Pacific to Oceania. Differently from the reports by⁴⁻⁸, which found a close relationship between *Eleotris amblyopsis* and *Eleotris fusca*, the new taxa included in our phylogenetic analyses indicated that *Eleotris amblyopsis* is the sister species of *Eleotris* sp. 2, a newly discovered lineage in Northern coast of Brazil³. We also recovered *B. gyrinoides* and *C. godeffroyi* within the clade *Eleotris/ Erotelis*. Both species are distributed in the Indo-Pacific region and have a disjunct range when compared to the Neotropical genera *Eleotris* and *Erotelis*. Based on the presence of 10 + 15 vertebrae and pterygiophores in the first dorsal fin arranged in combination 3 (1221), the genera *Eleotris*, *Erotelis* and *Calumia* had been referred to the group "*Eleotris*"⁵³, which also includes freshwater and estuarine species of *Belobranchus* from Indo-Pacific. Yet, robust approaches to recover the evolutionary diversification and their relationships with past environmental conditions are required to elucidate the intriguing evolutionary history of such genera of eleotrids. For example, the role of dispersal and/or vicariance events on the distribution and phylogeographic structure of these species should be carefully investigated.

Our data revealed a close phylogenetic relationship between the genera *Guavina* and *Dormitator*, which has also been reported in the previous studies^{4,8,9}. Morphological evidence also supports these results inasmuch as both genera share one unambiguous synapomorphy first two hemal spines curved, arched; see⁵³. On the other hand, *D. latifrons* and *D. maculatus* were not recovered as sister species, thus differing from previous reports^{5,8,52}. The inclusion of new species in this study, i.e., *D. lebretonis* and *D. cubanus* resulted in a close relationship between species from the Atlantic Ocean, following the same trend observed in the diversification of *Eleotris*, in which the Atlantic species (*D. maculatus*, *D. cubanus* and *D. lebretonis*) form a monophyletic group. Therefore, our results corroborate the previous inference by³⁹.

According to the present molecular analyses, *Gobiomorus* is paraphyletic in relation to *H. latifasciata*, as also indicated by⁸. *Gobiomorus dormitor* (Western Atlantic) and *G. polylepis* (Eastern Pacific) are also sister species, representing a didactic example of geminate species that diverged after the formation of the Isthmus of Panama. The origin of Eleotridae dates to Eocene (55.6 My), but their ancestral area remains unknown because sister groups to this family were not included in this study. Our phylogenetic analysis successfully recovered the species from Eastern Pacific and Western Atlantic (*Erotelis armiger* / *E. smaragdus*; *Guavina guavina* / *G. micropus*; *Gobiomorus polylepis* / *G. dormitor*, *Leptophilypnus fluviatilis* / *L. panamesis*) as sister groups, similarly to the results obtained by⁵². The time-calibrated phylogeny showed that the lineages diverged before the formation of the Isthmus of Panama 3.1 My;⁵⁴. However, the most recent speciation events (1.4 Ma) occurred between *D. cubanus*, endemic to Cuba, and *D. lebretonis* from Eastern-Central Atlantic.

Regarding the miniaturized clade *Microphilypnus*, the estimate of divergence time of the clade *Dormitator* / *Guavina* was approximately 16.6 Mya. ⁵⁵considered *M. ternetzi*, *Dormitator* and *Eleotris* as marine-derived taxa, representing an endemic remnant of ancient radiations to Neotropical freshwater habitats. This result is in accordance with the origin of freshwater lineages from marine ancestors driven by sea level fluctuations in South America coast during Cretaceous-Eocene^{55,56,57}. Thacker Many clades, such as *Plagioscion* (Sciaenidae), *Jurengraulis* and *Anchovia* (Engraulidae), and *Pseudotylosurus* (Belonidae), have probably evolved from marine lineages by the connections formed between the Caribbean Sea and the Upper Amazon basin during this period via Los Llanos basin and Pebas Lake in Venezuela^{58,59,60,61,62}.

Similarly, the paraphyletic status of *Gobiomorphus* in relation to *Hemieleotris* herein observed agrees with other reports. However, we suggest caution before the synonymization of these lineages since unambiguous synapomorphies for the clade *Hemieleotris* and *G. polylepis* / *G. dormitor* have not been described. In this context, subsequent radiations throughout the South American basins determined a profusion of morphologically and ecologically distinct species not seen in marine habitats⁶³. Exploring this biogeographic background is highly recommended to understand the miniaturization process in Eleotridae. In fact, smaller body sizes in freshwater taxa when compared to marine forms have been widely reported, with explanations ranging from the advantages of reduced size in offering greater

maneuverability in structured environments⁶⁴ to the reduction of energetic demands in size-constrained or complex microhabitats²⁸.

In summary, these data provide the most complete hypothesis for Eleotridae phylogeny to date, because it includes representatives from several biogeographical regions. Our results were based on evolutionary information from mitochondrial and nuclear genes, and then, revealed a novel phylogenetic relationship from previous studies based only on mtDNA. The miniaturization does not seem to be a frequent event in Eleotridae, because the miniature taxa evolved in at least two genera (*Microphilypnus* and *Leptophilypnion*). As a result, we propose that miniaturization is an evolutionary process in the genus *Microphilypnus* with a strongly supported sister group relationship between *Microphilypnus* and the neotropical genus *Guavina*, *Dormitator* and *Gobiomorus*. As the position of *Leptophilypnion* was not established in the phylogeny, we cannot affirm the close relationship between the miniature taxa. Thus, more extensive taxonomic and geographical sampling and analysis based on multi loci may reveal whether this event is exclusively part of a clade. The non-miniature genus *Leptophilypnus* was often considered to be a sister group of the *Microphilypnus*, however, our results are consistent with the hypothesis that both lineages evolved independently.

Material And Methods

Taxon sampling. A total of 48 samples were included in the phylogenetic analyses, being 22 of them collected in the wild and 26 obtained from GenBank (Table S1). The dataset consisted of 22 species of Eleotridae found exclusively in Neotropical region, including members of all currently recognized genera, except *Leptophilypnion*³⁴, which has a relatively recent description with no genetic data available in public databases. To explore the phylogenetic relationships hypothesized in previous studies^{4,8,39}, we also included DNA sequences of species from other biogeographical regions, such as Indo-Pacific, Australia, New Zealand, New Guinea, Madagascar, and Africa. Four species of Gobiiformes (*Perccottus glenii*, *Odontobutis potamophila*, *Odontobutis obscura* and *Rhyacichthys aspro*) were used as outgroup. All newly acquired sequences were deposited in GenBank (accession numbers in Supplementary Material 1).

DNA Extraction, PCR, and genomic sequencing. Total genomic DNA was extracted from muscle tissue using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI - USA). The Polymerase Chain Reaction (PCR) was carried out to obtain 2,254 base pairs (bp) of three mitochondrial markers: ~ 565 bp of 16S rRNA gene (*16S*); ~ 697 bp of cytochrome c oxidase I (*COI*); ~ 992 bp of NADH dehydrogenase subunit 2 (*ND2*); and 800 pb of two single-copy exons: ~ 429 bp of rhodopsin gene (*RHOD*); ~ 371 bp of early growth response 1 (*EGR1*). The amplification reactions were performed in a final volume of 25 µL, containing 4 µl of the dNTP (1.25 mM), 2.5 µl of 10x buffer solution, 1 µl of MgCl₂ (25 Mm), 0.25 µl of each primer (200 ng/µl), 1 µl of template DNA (100 ng/µl), 1 µl of Taq DNA polymerase (5 U/µl) and 15 µL of ultrapure water.

For the mtDNA markers, the PCRs conditions were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of 40s at 94°C, 40s of annealing, 72°C for 3 min, and a final extension of 5 min at 72°C. The amplification conditions for nuDNA included an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 40s for denaturation, 30s for annealing, and 72°C for 90 s for extension, plus a final extension of 7 min at 72°C. The efficiency of amplification via PCR was checked in a 2% agarose gel. Amplified products were purified with PEG (polyethylene glycol) and sequencing reactions were performed with the BigDye reagent kit. The purified samples were then sequenced by the Sanger method⁴⁰ using an ABC 3500xL automatic sequencer (Applied Biosystems).

Phylogenetic analyses. The sequences were aligned automatically using MUSCLE⁴¹, as implemented in GENEIOUS 9.0.5 (<https://www.geneious.com>). The phylogenetic analyses were performed based on concatenated mitochondrial and nuclear partitions but applying separate priors. The aligned sequences of multiple loci were concatenated using SequenceMatrix 1.7.8⁴². The best-fit evolutionary model was selected in PartitionFinder 2 [43] for each gene and for each codon position in the case of protein-coding genes. The best-fit partitioning schemes and models are shown in Supplementary material 2.

Estimates of divergence times and species tree. The analysis of TMRCA (Time of the Most Recent Common Ancestral) as well as a species tree (*BEAST)⁴⁴ were implemented in *BEAST 2.5.2⁴⁵. We used the five genes according to the optimal partitioning strategy as indicated by PartitionFinder 2 (Table S2). The simulations were carried out assuming an uncorrelated lognormal relaxed molecular clock, and the Yule speciation process as a prior⁴⁶. The BEAST analysis comprised two independent runs, using 10×10^7 generations, sampled every 5000 generations. The first 10% of all samples were removed as burn-in, and Tracer 1.7.1⁴⁷ was used to check the effective samples sizes (ESS) assuming optimal parameters (> 200). The maximum credibility tree was generated in TreeAnnotator v1.6.1⁴⁸. The resulting phylogenetic trees were visualized in Figtree 1.4.3⁴⁹. The TMRCA was estimated based on the recovered ages in the study developed by⁵⁰. These authors calibrated points from the fossil record using a subset of 202 taxa, 18 genes, and 59 calibration points. Based on this study, we used the origin of the family Eleotridae (mean age of 55.47 Ma) as a calibration point.

Declarations

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Data availability

The datasets generated and analyzed in the current study are available in GenBank (GenBank accession numbers shown in supplementary material).

References

1. Nelson, J. S., Grande, T. C., Wilson, M.V., 2016. *Fishes of the World*. John Wiley & Sons, New Jersey.
2. Fricke, R., Eschmeyer, W. N. & Van der Laan, R. (eds) 2021. *Eschmeyer's Catalog of fishes: Genera, Species, references*. (<http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp>). Electronic version accessed 12 February 2021.
3. Guimarães-Costa, A., Vallinoto, M., Giarrizzo, T., Pezold, F., Schneider, H., Sampaio, I., 2016. **Molecular evidence of two new species of *Eleotris* (Gobiiformes: Eleotridae) in the western Atlantic**. *Mol. Phylogenet. Evol.* **98**:52–56. <https://doi.org/10.1016/j.ympev.2016.01.014>
4. Thacker, C.E., Hardman, M.A., 2005. Molecular phylogeny of basal gobioid fishes: Rhyacichthyidae, Odontobutidae, Xenisthmidae, Eleotridae (Teleostei: Perciformes: Gobioidae). *Mol. Phylogenet. Evol.* **37**:858–887. [10.1016/j.ympev.2005.05.004](https://doi.org/10.1016/j.ympev.2005.05.004)
5. Nordlie, F.G., 2012. Life-history characteristics of eleotrid fishes of the western hemisphere, and perils of life in a vanishing environment. *Rev. Fish Biol. Fisher.* **22**(1), 189–224. <https://doi.org/10.1007/s11160-011-9229-3>
6. Berra, T.M., 2001. *Freshwater fish distribution*. Academic Press, San Diego.
7. Graham, J.B., 1997. *Air-breathing fishes: evolution, diversity, and adaptation*. Academic Press, San Diego.
8. Thacker, C.E., 2009. Phylogeny of Gobioidae and its placement within Acanthomorpha, with a new classification and investigation of diversification and character evolution. *Copeia*. **1**:93–104. [10.1643/CI-08-004](https://doi.org/10.1643/CI-08-004)
9. Chakrabarty, P., Davis, M.P., Sparks, J.S., 2012. The first record of a trans-oceanic sister-group relationship between obligate vertebrate troglobites. *PLoS ONE* **7**:e44083. <https://doi.org/10.1371/journal.pone.0044083>
10. Karl, S.A., Avise, J.C., 1992. Balancing selection at allozyme loci in oysters: implications from nuclear RFLPs. *Science*. **256**:100. [10.1126/science.1348870](https://doi.org/10.1126/science.1348870)
11. Hey, J., Machado, C.A., 2003. The study of structured populations - new hope for a difficult and divided science. *Nat. Rev. Genet.* **4**:535–543. <https://doi.org/10.1038/nrg1112>
12. Agorreta, A., San Mauro, D., Schlieven, U., Van Tassell, J.L., Kovacic, M., Zardoya, R., Rüber, L., 2013. Molecular phylogenetics of Gobioidae and phylogenetic placement of European gobies. *Mol. Phylogenet. Evol.* **69**:619–633. <https://doi.org/10.1016/j.ympev.2013.07.017>
13. Castroviejo-Fisher, S., Guayasamin, J.M., Gonzalez-Voyer, A., Vilà, C., 2014. Neotropical diversification seen through glassfrogs. *J. Biogeogr.* **41**:66–80. <https://doi.org/10.1111/jbi.12208>
14. Dornburg, A., Townsend, J.P., Friedman, M., Near, T.J., 2014. Phylogenetic informativeness reconciles ray-finned fish molecular divergence times. *BMC Evol. Biol.* **14**:169. [doi:10.1186/s12862-014-0169-0](https://doi.org/10.1186/s12862-014-0169-0)
15. Hundt, P.J., Iglésias, S.P., Hoey, A.S., Simons, A.M., 2014. A multilocus molecular phylogeny of combtooth blennies (Percomorpha: Blennioidei: Blenniidae): Multiple invasions of intertidal habitats.

- Mol. Phylogenet. Evol. 70:47–56. [10.1016/j.ympev.2013.09.001](https://doi.org/10.1016/j.ympev.2013.09.001)
16. Olave, M., Avila, L.J., Sites, J.W., Morando, M., 2014. Multilocus phylogeny of the widely distributed South American lizard clade *Eulaemus* (Liolaemini, Liolaemus). *Zool. Scr.* 43:323–337. <https://doi.org/10.1111/zsc.12053>
 17. Meyer, B.S., Matschiner, M., Salzburger, W., 2015. A tribal level phylogeny of Lake Tanganyika cichlid fishes based on a genomic multi-marker approach. *Mol. Phylogenet. Evol.* 83:56–71. <https://doi.org/10.1016/j.ympev.2014.10.009>
 18. Jønsson, K.A., Fabre, P.H., Kennedy, J.D., Holt, B.G., Borregaard, M.K., Rahbek, C., Fjeldså, J., 2016. A supermatrix phylogeny of corvoid passerine birds (Aves: Corvides). *Mol. Phylogenet. Evol.* 94:87–94. <https://doi.org/10.1016/j.ympev.2015.08.020>
 19. Li, H., Durbin, R., 2011. Inference of human population history from individual whole-genome sequences. *Nature.* 475(7357), 493–496. <https://doi.org/10.1038/nature10231>
 20. Frantz, R. S. (2013). *X-efficiency: Theory, evidence and applications* (Vol. 2). Springer Science & Business Media.
 21. Bessa-Silva, A., Vallinoto, M., Sampaio, I., Flores-Villela, O.A., Smith, E.N., Sequeira, F., 2020. The roles of vicariance and dispersal in the differentiation of two species of the *Rhinella marina* species complex. *Mol. Phylogenet. Evol.* 145, 106723. [10.1016/j.ympev.2019.106723](https://doi.org/10.1016/j.ympev.2019.106723)
 22. Leutenegger, W., 1973. Maternal-fetal weight relationships in primates. *Folia Primatol.* 20(4), 280–293. [10.1159/000155580](https://doi.org/10.1159/000155580)
 23. Yeh, J., 2002. The effect of miniaturized body size on skeletal morphology in frogs. *Evolution.* 56(3), 628–641. <https://doi.org/10.1111/j.0014-3820.2002.tb01372.x>
 24. Daza, J.D., Bauer, A.M., Stanley, E.L., Bolet, A., Dickson, B., Losos, J. B. (2018). An enigmatic miniaturized and attenuate whole lizard from the Mid-Cretaceous amber of Myanmar. *Breviora.* 563(1), 1–18. <https://doi.org/10.3099/MCZ49.1>
 25. Hanken, J., Wake, D.B., 1993. Miniaturization of body size: organismal consequences and evolutionary significance. *Annu. Rev. Ecol. Evol. Syst.* 24(1), 501–519. <https://doi.org/10.1146/annurev.es.24.110193.002441>
 26. Britz, R., Conway, K.W., 2009. Osteology of *Paedocypris*, a miniature and highly developmentally truncated fish (Teleostei: Ostariophysi: Cyprinidae). *J. Morphol.* 270 (4): 389–412. doi: [10.1002/jmor.10698](https://doi.org/10.1002/jmor.10698)
 27. Britz, R. Conway, K.W., Ruber, L., 2009. Spectacular morphological novelty in a miniature cyprinid fish, *Danionella dracula* n. sp. *Proc R Soc Lond [Biol]*. 276 (1665): 2179–2186. doi: [10.1098/rspb.2009.0141](https://doi.org/10.1098/rspb.2009.0141)
 28. Weitzman, S.H., Vari, R.P., 1988. Miniaturization in South American freshwater fishes; an overview and discussion. *Proc. Biol. Soc. Wash.* 101(2): 444–465.
 29. Toledo-Piza, M., Mattox, G. M., Britz, R., 2014. *Priocharax nanus*, a new miniature characid from the rio Negro, Amazon basin (Ostariophysi: Characiformes), with an updated list of miniature Neotropical freshwater fishes. *Neotrop. Ichthyol.* 12(2), 229–246. <https://doi.org/10.1590/1982-0224-20130171>

30. Caires, R.A., Figueiredo, J.L., 2011. Review of the genus *Microphilypnus* Myers, 1927 (Teleostei: Gobioidae: Eleotridae) from the lower Amazon basin, with description of one new species. *Zootaxa*. 3036:39–57. <https://doi.org/10.11646/zootaxa.3036.1.3>
31. Caires, R.A., 2013. *Microphilypnus tapajosensis*, a new species of eleotridid from the Tapajós basin, Brazil (Gobioidae: Eleotrididae). *Ichthyol. Explor. Freshw.* 23, 155–160.
32. Caires, R.A., Guimarães-Costa, A., 2017. Family Eleotridae. In: Peter van Sleen; James Albert. (Org.). *Field Guide to Amazonian Fishes*, first ed. Princeton University Press, New Jersey, pp. 388–391.
33. Caires, R.A., Toledo-Piza, M., 2018. A New Species of Miniature Fish of the Genus *Microphilypnus* (Gobioidae: Eleotridae) from the Upper Rio Negro Basin, Amazonas, Brazil. *Copeia*. 106(1), 49–55. [10.1643/CI-17-634](https://doi.org/10.1643/CI-17-634)
34. Roberts, T.R., 2013. *Leptophilypnion*, a new genus with two new species of tiny central Amazonian gobioid fishes (Teleostei, Eleotridae). *Aqua*.
35. Gould, R. E., & Delevoryas, T. (1977). The biology of *Glossopteris*: evidence from petrified seed-bearing and pollen-bearing organs. *Alcheringa*, 1(4), 387–399.
36. Rüber, L., Kottelat, M., Tan, H.H., Ng, P.K., Britz, R., 2007. Evolution of miniaturization and the phylogenetic position of *Paedocypris*, comprising the world's smallest vertebrate. *BMC Evol. Biol.* 7(1), 1–10. [10.1186/1471-2148-7-38](https://doi.org/10.1186/1471-2148-7-38)
37. Bloom, D. D., Kolmann, M., Foster, K., & Watrous, H. (2019). Mode of miniaturisation influences body shape evolution in New World anchovies (Engraulidae). *Journal of fish biology*, 96(1), 194–201.
38. Thacker, C.E., 2003. Molecular phylogeny of the gobioid fishes (Teleostei: Perciformes: Gobioidae). *Mol. Phylogenet. Evol.* 26:354–368. [https://doi.org/10.1016/S1055-7903\(02\)00361-5](https://doi.org/10.1016/S1055-7903(02)00361-5)
39. Galván-Quesada, S., Doadrio, I., Alda, F., Perdices, A., Reina, R. G., Varela, M. G., Hernández, N., Mendoza, A. C., Bermingham, E., Domínguez-Domínguez, O., 2016. Molecular phylogeny and biogeography of the amphidromous fish genus *Dormitator* Gill 1861 (Teleostei: Eleotridae). *PloS one*, 11(4), e0153538. <https://doi.org/10.1371/journal.pone.0153538>
40. Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *PNAS*. 74(12): 5463–5467. [10.1073/pnas.74.12.5463](https://doi.org/10.1073/pnas.74.12.5463)
41. Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32(5), 1792–1797. <https://doi.org/10.1093/nar/gkh340>
42. Vaidya, G., Lohman, D. J., & Meier, R. (2011). SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*, 27(2), 171–180.
43. Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., Calcott, B., 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* [dx.doi.org/10.1093/molbev/msw260](https://doi.org/10.1093/molbev/msw260)
44. Heled, J., Drummond, A.J., 2008. Bayesian inference of population size history from multiple loci. *BMC Evol. Biol.* 8(1), 1–15. <https://doi.org/10.1186/1471-2148-8-289>

45. Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C-H., Xie, D., Suchard, MA., Rambaut, A., Drummond, A.J., 2014. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Comput. Biol.* 10(4), e1003537. doi:10.1371/journal.pcbi.1003537
46. Drummond, A.J., Ho, S.Y., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. *PLoS Biol.* 4(5), e88. <https://doi.org/10.1371/journal.pbio.0040088>
47. Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst. Biol.* 67(5): 901. 10.1093/sysbio/syy032
48. Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7:214. <https://doi.org/10.1186/1471-2148-7-214>
49. Rambaut, A., 2017. FigTree, a graphical viewer of phylogenetic trees (Version 1.4. 3).
50. Betancur-R, R., Wiley, E.O., Arratia, G., Acero, A., Bailly, N., Miya, M., Leicontre, G., Orti, G. 2017. Phylogenetic classification of bony fishes. *BMC Evol. Biol.* 17(1), 1–40. 10.1186/s12862-017-0958-3
51. Britz, R., Conway, K. W., Rüber, L., 2014. Miniatures, morphology and molecules: *Paedocypris* and its phylogenetic position (Teleostei, Cypriniformes). *Zool. J. Linnean Soc.* 172(3), 556–615. <https://doi.org/10.1111/zoj.12184>
52. Thacker, C.E., 2017. Patterns of divergence in fish species separated by the Isthmus of Panama. *BMC Evol. Biol.* 17(1), 1–14. <https://doi.org/10.1186/s12862-017-0957-4>
53. Birdsong, R.S., Murdy, E.O., Pezold, F.L., 1988. A study of the vertebral column and median fin osteology in gobioid fishes with comments on gobioid relationships. *Bull. Mar. Sci.* 42 (2): 174–214.
54. Lessios, H.A., 2008. The great American schism: divergence of marine organisms after therise of the central American isthmus. *Annu. Rev. Ecol. Evol. Syst.* 2008; 39:63–92. <https://doi.org/10.1146/annurev.ecolsys.38.091206.095815>
55. Lovejoy, N.R., Albert, J.S., Crampton, W.G., 2006. Miocene marine incursions and marine/freshwater transitions: Evidence from Neotropical fishes. *J. South Am. Earth Sci.* 21:5–13. <https://doi.org/10.1016/j.jsames.2005.07.009>
56. Cooke, G.M., Chao, N.L., Beheregaray, L.B., 2012. Marine incursions, cryptic species and ecological diversification in Amazonia: the biogeographic history of the croaker genus *Plagioscion* (Sciaenidae). *J. Biogeogr.* 39:724–738. <https://doi.org/10.1111/j.1365-2699.2011.02635.x>
57. Bloom, D.D., Lovejoy, N.R., 2017. On the origins of marine-derived freshwater fishes in South America. *J. Biogeogr.* 44(9), 1927–1938. <https://doi.org/10.1111/jbi.12954>
58. Monsch, K.A., 1998. Miocene fish faunas from the northwestern Amazonia basin (Colombia, Peru, Brazil) with evidence of marine incursions. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 143:31–50. [https://doi.org/10.1016/S0031-0182\(98\)00064-9](https://doi.org/10.1016/S0031-0182(98)00064-9)
59. Hoorn, C., 1993. **Marine incursions and the influence of Andean tectonics on the Miocene depositional history of northwestern Amazonia: results of a palynostratigraphic study.** *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 105:267–309. [https://doi.org/10.1016/0031-0182\(93\)90087-Y](https://doi.org/10.1016/0031-0182(93)90087-Y)

60. Hoorn, C., Guerrero, J., Sarmiento, G.A., Lorente, M.A., 1995. **Andean tectonics as a cause for changing drainage patterns in Miocene northern South America**. *Geology*. **23**:237–240. [https://doi.org/10.1130/0091-7613\(1995\)023%3C0237:ATAACF%3E2.3.CO;2](https://doi.org/10.1130/0091-7613(1995)023%3C0237:ATAACF%3E2.3.CO;2)
61. Gingras, M.K., Rasanen, M.E., Pemberton, S.G., Romero, L.P., 2002. Ichnology and sedimentology reveal depositional characteristics of bay-margin parasequences in the Miocene Amazonian foreland basin. *J Sediment Res.* **72**:871–883. <https://doi.org/10.1306/052002720871>
62. Wesselingh, F.P., Rasänen, M.E., Irion, G., Vonhof, H.B., Kaandorp, R., Renema, W., Pitmann, L.R., Gingras, M., 2002. Lake Pebas: a palaeoecological reconstruction of a Miocene, long-lived lake complex in western Amazonia. *Cainoz. Res.* **1**, 35–81.
63. Bloom, D. D., & Lovejoy, N. R. (2012). Molecular phylogenetics reveals a pattern of biome conservatism in New World anchovies (family Engraulidae). *Journal of Evolutionary Biology*, *25*(4), 701–715.
64. Ward, A.B., Azizi, E., 2004. Convergent evolution of the head retraction escape response in elongate fishes and amphibians. *Zoology*. **107**(3): 205–217. <https://doi.org/10.1016/j.zool.2004.04.003>

Figures

Figure 1

Previous phylogenetic hypotheses among eleotrids based on mitochondrial genes. Note the different positions of clades *Microphilypnus* (green), *Leptophilypnus* (blue), *Philypnodon* (red), and *Calumia* (yellow).

Figure 2

Combined mitochondrial and nuclear DNA species tree based on the algorithm implemented in *BEAST. Blue bars illustrate the 95% highest posterior density of node heights and support values of the posterior probability are displayed on each node. The clade in green shows the phylogenetic position of the miniature lineage of *Microphilypnus*, while *Leptophilypnus* (red branch) appears as sister group of other eleotrids.

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