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Mitochondrial evidence on the phylogenetic position of the Southeast Asian catfish genus *Encheloclarias* Myers, 1937 (Actinopterygii: Siluriformes: Clariidae): Evolutionary and conservation implications

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Abstract

The phylogenetic position of the Southeast Asian catfish genus *Encheloclarias* Myers, 1937 within the family Clariidae is inferred herein using three mitochondrial nucleotide markers: *cytochrome b*, *cytochrome oxidase subunit I*, and *16S* rRNA genes. We found that *Encheloclarias* is neither exclusively related to the African taxa having extended neural spines posterior to the dorsal fin (*Encheloclarias* shares with some of these taxa the presence of an adipose fin, as opposed to absent in all other clariid taxa) nor to the Asian species of the genus *Clarias* Scopoli, 1777. *Encheloclarias* is hypothesized to be the sister group of all other clariids, except *Horaglanis* Menon, 1951. The inferred position of *Encheloclarias* confirms that the adipose fin in this genus has an evolutionary origin independent to that of the adipose fin found in some African clariids. *Encheloclarias* is not only ecologically remarkable, being adapted to acidic peat swamps in Southeast Asia, but it is also an ancient lineage sheltering in these habitats. However, the precise timing of the colonization of peat swamps by *Encheloclarias* remains to be investigated. The phylogenetic position of *Encheloclarias* further underscores the importance of studying and protecting the remaining peat swamp habitats in Southeast Asia and their distinctive aquatic fauna.

Keywords

adaptation, adipose fin, convergence, molecular phylogenetics, peat swamp forests, Teleostei

Introduction

The Southeast Asian genus *Encheloclarias* was established by Myers in Herre and Myers (1937) to reclassify a clariid species originally described as *Heterobranchus tapeinopterus* Bleeker, 1853. *Encheloclarias* is one of three genera in the family Clariidae (walking catfishes) occurring in Asia, alongside *Clarias* Scopoli, 1777 and *Horaglanis* Menon, 1951. The Clariidae is otherwise distributed in Africa, where it exhibits the greatest generic

diversity with 14 genera (including *Clarias*, which is found in both Asia and Africa). There are currently six valid species of *Encheloclarias* (see Fricke et al. 2024), most of which are known from only a few specimens, indicating their possible rarity (Ng and Lim 1993; Tan et al. 2023). *Encheloclarias* species inhabit acidic peat swamp habitats of Southeast Asia (Malaysia, Singapore, Indonesia, and Brunei), where they burrow into the peat soil layer (Ng and Lim 1993; Ng and Tan 2000). The smallest species is *Encheloclarias baculum* Ng et Lim, 1993 (5.7 cm reported maximum SL), while the largest is *Encheloclarias velatus* Ng et Tan, 2000 (16.9 cm reported maximum SL). There is still limited information on the biology, morphology (especially osteology), and evolution of this genus (Ng and Lim 1993; Teugels and Adriaens 2003; Tan et al. 2023). The phylogenetic position of this genus is virtually unknown.

Within the Clariidae, *Encheloclarias* possesses an adipose fin (Fig. 1), a feature it shares with only few African taxa such as *Heterobranchus* Geoffroy St. Hilaire, 1809, *Dinotopterus* Boulenger, 1906, and *Clarias ngamensis* Castelnau, 1861. Although the adipose fin is externally similar between these African taxa and *Encheloclarias*, its structure differs between them (Teugels and Adriaens 2003). The adipose fin in African taxa is supported by elongated neural spines while there is no osteological support in that of *Encheloclarias*. Therefore, the presence of an adipose fin in African taxa and *Encheloclarias* is not considered an evidence of their close relationship. Teugels and Adriaens (2003) wrote "Based on zoogeographical evidence, *Horaglanis* and *Encheloclarias* probably descended independently from Asian *Clarias*." Subsequent phylogenetic works on clariids, including those by Devaere et al. (2007) and Pouyaud et al. (2009), did not include *Encheloclarias*.

The absence of phylogenetic information on *Encheloclarias* limits discussion on the evolution of the Clariidae. For example, the independent origins of the adipose fin in the Clariidae have not yet been confirmed and it is also unclear whether the presence of an adipose fin in *Encheloclarias* represents the ancestral or derived condition in this family (Stewart et al. 2014).

In this work, we infer the phylogenetic position of *Encheloclarias* by reconstructing the phylogeny of 13 (out of 16) clariid genera using three mitochondrial markers: the *cytochrome b* (*cytb*), *cytochrome oxidase subunit I* (*COI*), and *16S rRNA* (*16S*) genes.

Materials and methods

Specimen collection and molecular sampling. Fragments of the *cytb* (~600 bp), *COI* (~655 bp), and *16S* (~700 bp) nucleotide sequences from three specimens of *Encheloclarias curtisoma* Ng et Lim, 1993 were newly determined for this study. Specimens were collected using dipnets from three peat swamp forests (Pondok Tanjung, North Selangor, and Ayer Hitam) in West Peninsular Malaysia. A tissue sample from each specimen was excised after euthanasia by rapid cooling in ice-water after capture, a procedure recommended by Blessing et al. (2010), then preserved in 95% ethanol, and finally stored at room temperature. Specimens were formalin-fixed in a 10% formalin solution for two weeks, then rinsed with tap water before being transferred into 70% ethanol. All three specimens are deposited in the Makmal Rujukan Zoologi (Zoological Reference Laboratory), Universiti Sains Malaysia (USMFC), under the following catalogue numbers USMFC (19) 00031 (locality: Pondok Tanjung; specimen code: APT57), USMFC (19) 00032 (North Selangor; BNS79), and USMFC (19) 00033 (Ayer Hitam; JAH37).

The molecular dataset was completed by selecting additional *cytb*, *COI*, and *16S* sequences available in GenBank representing a total of (alongside *Encheloclarias curtisoma*) 12 genera and 28 species of the Clariidae (Table 1). The three missing clariid genera are the African *Uegitglanis* Gianferrari, 1923, *Platyclarias* Poll, 1977, and *Xenoclarias* Greenwood, 1958. *Heteropneustes fossilis* (Bloch, 1794) (family Heteropneustidae) which is closely related to the Clariidae (see Diogo et al. 2003; Sullivan et al. 2006), was included in the dataset along with a more distantly catfish outgroup, *Occidentarius platypogon* (Günther, 1864) (family Ariidae).

Figure 1. Photo of a live specimen of *Encheloclarias curtisoma* (Clariidae) from the locality Ayer Hitam (Johor, Peninsular Malaysia) showing the dorsal fin and the adipose fin.

DNA extraction, PCR amplification, and sequencing. Total genomic DNA was extracted from the fin clip using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Grewe et al. 1993). Each gene was separately amplified by PCR amplification carried out with a T100™ Thermal Cycler (BIO-RAD, Hercules, California, USA). A 655 base pairs (bp) long fragment *COI* gene was amplified using the primer set FishF1 (5′–TCA ACC AAC CAC AAA GAC ATT GGC AC–3′) and FishR1 (5′–TAG ACT TCT GGG TGG CCA AAG AAT CA–3′) (Ward et al. 2005). The partial *cytb* gene (~600 bp) was amplified using the following primer set: L15267 (5′–AAT GAC TTG AAG AAC CAC CGT–3′) and H15891 (5′–GTT TGA TCC CGT TTC GTG TA–3′) (Briolay et al. 1998). A ~700 bp long fragment of the *16S* gene was amplified using the primer set 16S_F (5′–CTC GTA CCT TTT GCA TCA TG–3′) and 16S_R (5′–AAG TGA TTG CGC TAC CTT TG–3′) (Pouyaud et al. 2009). Each PCR mixture was carried out in a total volume of 25 µL, which contained 10.5 µL of ddH2O, 12.5 µL of 2X MyTaq™ Red Mix buffer (Meridian Bioscience, Ohio, USA), 0.5 µL of each 10 µM primer, and 1.0 µL of genomic DNA.

The PCR for *COI* gene was carried out under following thermal cycling conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 47.9°C for 50 s, primer extension at 72°C for 1 min and final extension for 7 min at 72°C. The PCR for *cytb* and *16S* genes was carried out following the protocol of Pouyaud et al. (2009). Successful PCR samples were sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for purification and bidirectional sequencing using Sanger technology using the same PCR primers.

Sequence editing alignment procedure and phylogenetic reconstruction. Chromatograms were edited and the consensus sequence for each gene and specimen of *Encheloclarias curtisoma* was built by assembling the forward and reverse sequences using MEGA v11.0 (Tamura et al. 2021). Sequences were deposited in GenBank under accession numbers PP273447-PP273449 (*cytb*), and PP274029 and PP274030 (*16S*) and in BOLD under accession numbers NCTF1312-24, NCTF757-24, and NCTF799-24 (*COI*), (Table 1). Nucleotide sequences of *cytb* and *COI* were separately aligned by hand, and

Table 1. List of species of the Clariidae examined in this study along with information on specimens and molecular markers used. Bold GenBank and BOLD accession numbers indicate sequences determined in presently reported study.

Species	Specimen code	GenBank and BOLD accession numbers		
		cytb	*COI*	*16S*
Asian species				
Encheloclarias curtisoma Ng et Lim, 1993	APT57	**PP273447**	**NCTF1312-24**	**PP274029**
	BNS79	**PP273448**	**NCTF757-24**	**PP274030**
	JAH37	**PP273449**	**NCTF799-24**	—
Clarias fuscus (Lacepède, 1803)	—	KM029965	KM029965	KM029965
Clarias macrocephalus Günther, 1864	—	MT109097	MT109097	MT109097
Clarias batrachus (Linnaeus, 1758)	—	KC572134	KC572134	KC572134
Clarias dussumieri Valenciennes, 1840	—	MG644387	MG644387	MG644387
Clarias punctatus (Linnaeus, 1758)	IRD 1986	MW012844	—	MW012795
Clarias kapuasensis Sudarto, Teugels et Pouyaud, 2003	IRD 4678	MW012799	—	MW012775
Clarias leiacanthus Bleeker, 1851	IRD 4464	MW012805	—	MW012776
Clarias nieuhofii Valenciennes, 1840	MNHN 2003-0295(615)	MW012829	—	MW012788
Clarias olivaceus Fowler, 1904	IRD 4901	MW012833	—	MW012789
Clarias planiceps Ng, 1999	IRD 2127	MW012837	—	MW012791
Clarias pseudoleiacanthus Sudarto, Teugels et Pouyaud, 2003	ZRC 47145(4548)	MW012838	—	MW012792
Clarias pseudonieuhofii Sudarto, Teugels et Pouyaud, 2004	IRD 4664	MW012840	—	MW012793
Horaglanis krishnai Menon, 1951	—	OP832214	OP825111	OP824400
Horaglanis abdulkalami Babu, 2012	—	OP832203	OP825094	OP824386
African species				
Clarias camerunensis Lönnberg, 1895	—	OP936082	OP936082	OP936082
Clarias gariepinus (Burchell, 1822)	—	KT001082	KT001082	KT001082
Clarias gabonensis Günther, 1867	—	AY995129	—	—
Bathyclarias gigas Jackson, 1959	—	AF235928	—	—
Channallabes apus (Günther, 1873)	—	AF126820	—	—
Clariallabes longicauda (Boulenger, 1902)	—	AY995124	—	—
Dinotopterus cunningtoni Boulenger, 1906	—	AY995126	—	—
Gymnallabes typus Günther, 1867	—	AY995132	—	—
Heterobranchus longifilis Valenciennes, 1840	—	AF126828	—	—
Heterobranchus bidorsalis Geoffroy St. Hilaire, 1809	—	AF126825	—	—
Dolichallabes microphthalmus Poll, 1942	—	JF262202	—	—
Platyallabes tihoni (Poll, 1944)	—	JF297961	—	—
Tanganikallabes mortiauxi Poll, 1943	—	JF297962	—	—
Pseudotanganikallabes prognatha Wright, 2017	SAIAB 80226	KF650734	—	—
Outgroups				
Heteropneustes fossilis (Bloch, 1794)	—	AP012013	AP012013	AP012013
Occidentarius platypogon (Günther, 1864)	—	KY930717	KY930717	KY930717

cytb = cytochrome b, *COI = cytochrome oxidase I*, *16S = 16S rRNA*.

no alignment required any indels. Sequences of *16S* gene were automatically aligned using MAFFT with all parameter selection left as "default" (Katoh et al. 2019).

Separate phylogenetic analyses were first conducted on each individual marker dataset which allow to compare their respective quantity and quality of phylogenetic signal and detect possible topological incongruence. In the absence of supported incongruence, we then combined all three mitochondrial genes together. The total alignment comprises 3107 nucleotide positions. Phylogenetic analyses employed Maximum Likelihood (ML) and Bayesian inference.

The ML tree was built using the software RAxML-NG (Kozlov et al. 2019) as implemented in the graphical interface raxmlGUI 2.0 (Edler et al. 2021). Mitochondrial data were first divided into four partitions: the non-coding *16S* gene and the first, second, and third codon positions of the combined *cytb* and *COI* protein-coding genes. The best models of nucleotide substitution for each of these partitions was selected with ModelTest-NG (Darriba et al. 2020) (as implemented in RAxML-NG) as K80 + Γ, HKY, TN93 + Γ, and GTR + Γ, respectively. Bootstrap proportions were calculated (1000 replicates) to assess the robustness of each node. The trees were visualized with FigTree v1.4.4 (Rambaut 2018).

A time-calibrated Bayesian phylogenetic tree was inferred under a relaxed molecular clock in the BEAST2 version 2.6.4 suite (Bouckaert et al. 2019). For this analysis, *Horaglanis* and the outgroup *Occidentarius platypogon* were excluded. We used the same four partitions and the same models of sequence evolution as for the ML analysis, except for the first codon positions partition's model which was changed from K80 + Γ to the most similar one, JC69 + Γ (this is because K80 is not implemented in BEAST2). A Relaxed Clock Log Normal model and a Birth and Death tree model were selected. The age of the most recent common ancestor of the Heteropneustidae and the Clariidae was constrained to 50 million years old which corresponds to the maximum age of the oldest clariid fossil excavated (Gayet 1987). Two independent MCMC runs were carried out, each for 100 million generations, and parameter values and trees were sampled every 50 thousand generations. The log files generated by BEAST2 from each run were visualized with Tracer v1.7.1 (Rambaut et al. 2018) to confirm that analyses reached convergence. Twenty-five percent of the resulting trees of each run were discarded as burn-in before combining the remaining trees into a single file and calculating the maximum credibility consensus tree and mean node ages. The resulting time-calibrated tree was visualized and exported with FigTree v1.4.4.

Results

Our mitochondrial dataset includes three molecular markers for 29 species of the Clariidae, currently classified into 13 genera. The total amount of missing data reaches approximately 48% but it neither affected the tree topology, which was stable across the different analyses, nor the robustness of the nodes of interest, relative to the phylogenetic position of *Encheloclarias*, that are all supported by high statistical values.

The Maximum Likelihood (ML) phylogenetic tree of the family Clariidae, including *Horaglanis*, is visualized in Suppl. material 1. This tree is fully resolved with several relationships supported by Bootstrap Proportion (BP) values greater than 80%. Branch length heterogeneity is detected in this reconstruction, with the genus *Horaglanis* having a much longer branch than any other taxa. In this tree, the Clariidae is not recovered as monophyletic because *Horaglanis* is found to be the sister group to the clade comprising the Heteropneustidae and the rest of the Clariidae (Suppl. material 1). Although the phylogenetic hypothesis of the *Horaglanis* outside the Clariidae has already been proposed based on morphology (e.g., Diogo et al. 2003), its long branch (due to a fast mitochondrial rate of substitutions) could mislead the inference in our tree (Bergsten 2005). Because of that, and because *Horaglanis* is not the main object of this study, we removed *Horaglanis* from the dataset for the subsequent analyses. The exclusion of *Horaglanis* does not affect the other phylogenetic relationships, especially the position of *Encheloclarias* as shown in the ML tree and in the time-calibrated Bayesian tree (Fig. 2A and Fig. 2B, respectively).

In both ML and Bayesian trees (Fig. 2A, 2B), the species-rich genus *Clarias* is paraphyletic, with all African genera nested within it. African clariids form a monophyletic group (BP = 80%, Posterior probability [PP] = 1) that is the sister group to *Clarias dussumieri* Valenciennes, 1840, a south Asian (Indian subcontinental region) species. Other species of Asian *Clarias* form a monophyletic group (BP = 79%, PP = 1) that is the sister group to the clade comprising the African clariids and *Clarias dussumieri* (BP = 85%, PP = 1). The African species having elongated neural spines form also a monophyletic group (BP < 80%, PP = 1). In all reconstructions, *Encheloclarias* is consistently inferred as the sister group to all other clariid genera (except *Horaglanis* when it is included in the analysis; see Suppl. material 1 and explanations above).

In the time-calibrated Bayesian tree (Fig. 2B), the time divergence between the *Encheloclarias* and the rest of the clariids is estimated to 46.8 million years [95% Credibility Interval [CI] = 50.0–41.4 million years] whereas the mean age of the crown group *Encheloclarias curtisoma* is estimated to 5.1 million years old [95% CI = 7.8–2.8 million years old].

Discussion

The phylogenetic position of *Encheloclarias*. Although some progress has been made in resolving the phylogeny of the Clariidae (see Teugels and Adriaens 2003; Devaere et al. 2007; Pouyaud et al. 2009; De Alwis et al. 2023), the phylogeny remains only partially resolved, partly because some genera are still understudied (i.e., *Uegitglanis*, *Platyclarias*, *Horaglanis*, and *Encheloclarias*) and their affinities remain elusive (Diogo et al. 2003; Teugels and Adriaens 2003; Devaere et al. 2007; Pouyaud et al. 2009).

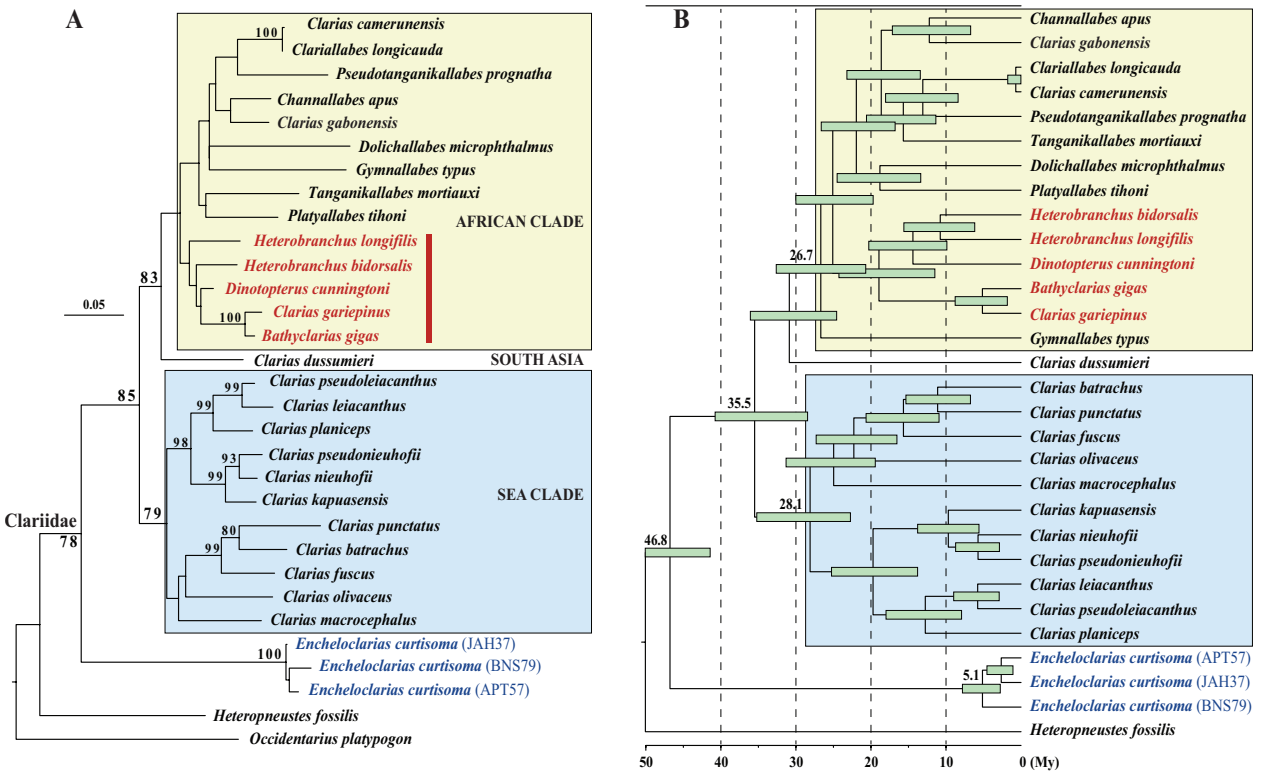

Figure 2. Phylogenetic trees of the family Clariidae showing the position of *Encheloclarias*. (**A**) ML Phylogenetic tree inferred using RAxML-NG. Branch lengths proportional to number of substitutions. Bootstrap Proportions shown at corresponding nodes if >75%. Names of the African species of Clariidae having extended neural spines posterior to dorsal fin (with or without an adipose fin) are highlighted in red. African and Southeast Asian (SEA) clades, comprising *Clarias* species, are highlighted in cream and blue, respectively. (**B**) Time-calibrated Bayesian phylogenetic tree inferred using BEAST2 v.2.6.4. Time scale in millions of years (My). Green bars at nodes indicate 95% Credibility Intervals for the ages of the corresponding nodes. Ages and posterior probability values for selected nodes indicated above and below, respectively. Dataset excludes *Horaglanis* because of its faster rate of molecular evolution relative to other taxa examined (see text for explanations).

This situation limits the understanding of the evolution of this group (e.g., Devaere et al. 2007, Stewart et al. 2014). Teugels and Adriaens (2003) summarized the phylogenetic information known at that time by presenting a morphology-based cladogram in which the African clariids (except *Uegitglanis*) and the Asian clariids (i.e., Asian *Clarias*, *Horaglanis*, and *Encheloclarias*) are reciprocally monophyletic. The position of *Uegitglanis* was left unresolved.

Using genetic data, we confirm herein the monophyly of the African clariids (Pouyaud et al. 2009), along with the monophyly of African taxa having elongated neural spines (indicated in red in Fig. 2A, 2B) within the African clariids (Devaere et al. 2007). Recently, De Alwis et al. (2023) inferred the non-monophyly of the Asian *Clarias* because South Asian *Clarias dussumieri* was found to be more closely related to African clariids than to Southeast Asian *Clarias*. A result replicated in our study. None of the previous genetic studies included *Encheloclarias*.

Herein, we infer that *Encheloclarias* is the sister group of the remaining clariids, not considering *Horaglanis* that we excluded from our analysis because the inference of the phylogenetic position of *Horaglanis* using only mitochondrial data may be unreliable due to its faster rate of molecular evolution. This hypothesis seems to not have been proposed before. Two previous hypotheses suggesting that *Encheloclarias* is closely related either to some African taxa such as *Heterobranchus*, *Dinotopterus*, and *Clarias ngamensis* (based on the shared presence of an adipose fin in these taxa) or to the Asian *Clarias* (as suggested by Teugels and Adriaens 2003) are therefore rejected. The inferred phylogenetic position of *Encheloclarias* has evolutionary and conservation implications that are briefly discussed below.

Evolution of the adipose fin in the Clariidae. Stewart et al. (2014) examined the evolution of the adipose fin in fish (Teleostei), highlighting the case of the Clariidae, in which most taxa possess only one long dorsal fin, apart from few taxa that have one dorsal fin and an adipose fin. Stewart et al. (2014) expressed uncertainty regarding the evolution of the adipose fin in the Clariidae, specifically whether the presence of an adipose fin represents the ancestral condition in this family. They lamented the lack of phylogenetic information on *Encheloclarias*.

Our phylogenetic results imply that the adipose fin in *Encheloclarias* and African taxa (such as *Heterobranchus*, and *Dinotopterus*) is not homologous, having evolved independently twice. This conclusion is also supported by the observation that the structure of the adipose fins in these two lineages differs. In African taxa, the adipose fin is supported by elongated neural spines, whereas in

Encheloclarias, it is not (Teugels and Adriaens 2003). Moreover, the early divergence of *Encheloclarias* does not allow us to dismiss the possibility that the adipose fin condition in *Encheloclarias* might represent the ancestral state for the Clariidae, as discussed by Stewart et al. (2014). However, the sole phylogenetic criterion is not sufficient to determine the ancestral condition of the dorsal and adipose fins at the origin of the Clariidae, given that three possibilities appear equally parsimonious: that of *Heteropneustes* with one short dorsal fin, that of *Encheloclarias* with one dorsal fin and one adipose fin, and that of the other clariids (excluding derived *Heterobranchus*, *Dinotopterus*, and *Clarias ngamensis*) with one long dorsal fin.

Conservation of *Encheloclarias* and their peat swamp habitats. Peat swamps in Southeast Asia are spatially dynamic environments known to harbor unique aquatic fauna, including many endemic fish species remarkably adapted to the highly acidic black waters (Ng et al. 1994; Fahmi-Ahmad et al. 2024). The evolution of these species remains poorly investigated. Genetic evidence suggests that some lineages adapted to peat swamp conditions millions of years ago, and subsequent allopatric diversification accounts for the observed diversity within these lineages (Fam et al. 2024). Nonetheless, additional data are required to assess the generality of this hypothesis and to determine whether the adaptation and diversification timeline is consistent across peat swamp lineages.

Encheloclarias represents one such lineage of fish adapted to the acidic peat swamps of Southeast Asia (Ng and Lim 1993). We estimated the mean age of divergence between *Encheloclarias* and the rest of the Clariidae to be 46.8 million years. This age is consistent with the timeline of the early diversification of the Clariidae proposed by Lundberg et al. (2007) based on a different set of calibrations and a different method of reconstruction. Thus, *Encheloclarias* is notable not only for its adaptation to acidic waters but also as an ancient and species-poor lineage. The genetic examination of other *Encheloclarias* species is needed to estimate the age of the crown group of the genus, and therefore to determine the minimum date of its colonization of peat swamps (herein, estimated to be only about 5 million years based on only one species, *Encheloclarias curtisoma*).

The possibility that the ancestors of *Encheloclarias* adapted to peat swamp environmental conditions millions of years ago further underscores the uniqueness of the fauna adapted to Southeast Asia's peat swamp forests. Yet, these remaining peat swamps continue to face severe threats.

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Supplementary material 1

Bayesian 50% consensus phylogenetic trees of the family Clariidae showing the position of *Encheloclarias*

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Data type: pdf

Explanation note: **A**) dataset including the genus *Horaglanis*; **B**) dataset excluding *Horaglanis* because of its higher rate of molecular evolution relative to other taxa examined (see text for details). Branch lengths proportional to number of substitutions. Posterior probabilities shown at corresponding nodes if < 1. Names of the species of Clariidae having two dorsal fins are highlighted in red. African and Southeast Asian (SEA) clades are highlighted in cream and blue, respectively.

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