

CHARLES UNIVERSITY

Faculty of Science

Department of Zoology

Study Program: Zoology



Mgr. Tomáš Dvořák

Phylogenetic, Biogeographic and Taxonomic investigations within the freshwater fish Family Nemacheilidae (Cypriniformes: Cobitoidea)

Rekonstrukce fylogenetických vztahů, biogeografie a taxonomie sladkovodních ryb z čeledi Nemacheilidae (Cypriniformes: Cobitoidea)

Doctoral thesis

Supervisor:

Dr. Jörg Bohlen, Ph.D.

Consultants:

Mgr. Vendula Bohlen Šlechtová, Ph.D.

Mgr. Zuzana Musilová, Ph.D.

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Author statement

I hereby declare that this PhD thesis is exclusively my own work, and that it has not been submitted (or any of its part) in order to obtain any academic degree earlier or at another institution. All publications and other sources used in the thesis have been properly cited. My contribution to the research presented in the thesis is reflected by the order of authors of the included publications.

Prague, 15.10.2024
Tomáš Dvořák

Prohlášení

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Tomáš Dvořák

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Abstract

With over 800 species, the family Nemacheilidae is one of the biggest families of primary freshwater fishes. Distributed nearly across whole Eurasia and with two species in northeast Africa, the family offers a rich subject for phylogenetic, biogeographic and taxonomic investigations. This doctoral thesis aims to untangle phylogenetic relationships within Nemacheilidae at multiple taxonomic levels.

The thesis comprises five articles published in peer-reviewed journals and one preprint currently undergoing revision. The main article presented here as a preprint provides the most comprehensive phylogenetic overview of the family Nemacheilidae to date and first extensive reconstruction of its biogeographic history. The study covers most of the distribution area of the family and significant portion of its taxonomic diversity, including 36 of the 49 recognized genera and over 250 species. The results of the phylogenetic analyses reveal several polyphyletic species and genera and several undescribed taxa. Six major clades were identified within the family. The reconstructed biogeographic history highlights the influence of local as well as global tectonic, geological, geographical and climatic events on the evolutionary history of Nemacheilidae.

Three of the five published articles are focused on the phylogenetic reconstruction of specific species groups in western Southeast Asia. First of them investigated the *Paracanthocobitis zonalternans* species complex, examining its phylogeny and biogeographic history. Phylogenetic analyses revealed multiple distinct clades, with mostly allopatric distributions and limited cases of co-occurrence. A single potential hybridisation event was also detected. Time-calibrated analyses revealed a significant impact of a global sea level fluctuations on evolutionary history of this species complex.

The second article focused on the *Schistura robertsi/cincticauda* species complex; a group of fishes specialised for very tiny forest streams that often reduce to few puddles during dry periods. Phylogenetic analyses identified ten well defined, genetically isolated lineages, five corresponding to recognized species and five representing new taxa. Despite of co-occurrence on several localities, the lineages exhibited no evidence of hybridisation and were consistently distinguishable based on morphological characters.

The third article explored a group of species sharing a unique pigmentation pattern, hypothesized to have originated from a single evolutionary event. However, phylogenetic analyses revealed two distinct species groups. Notably, besides the species with the characteristic

pigmentation, the two lineages were intermixed with taxa having completely different pigmentation pattern and even representatives from different genera.

The fourth article presents a follow up description of a new species within the *P. zonalternans* species complex. This genetically isolated species exhibits unique morphological characters and co-occurs with other two clades without evidence of hybridisation.

The fifth article expanded upon *S. robertsi/cincticauda* species complex, formally describing five new species and providing an identification key for all eleven species of the complex.

Key words: Cypriniformes, Cobitoidea, Nemacheilidae, phylogeny, biogeography, taxonomy, evolutionary history, *Schistura*, *Paracanthocobitis*.

Abstrakt

Čeďel' Nemacheilidae je s více než 800 druhy jednou z největších primárně sladkovodních čeďelí ryb. Díky svému rozšíření téměř po celé Eurasii, s výjimkou dvou druhů žijících v severovýchodní Africe, poskytuje tato čeďel' bohatý subjekt pro rekonstrukci fylogenetických vztahů, biogeografie a taxonomie. Cílem této disertační práce je objasnit fylogenetické vztahy v rámci čeďeli Nemacheilidae na několika různých taxonomických úrovních.

Práce obsahuje pět publikací v impaktovaných časopisech a jeden článek aktuálně procházející recenzním řízením. Hlavní publikace, která je zde prezentována jako článek v recenzním řízení, nabízí zatím nejvíce ucelený fylogenetický přehled a první rozsáhlejší rekonstrukci biogeografické historie. Tato studie pokrývá většinu areálu rozšíření čeďeli a významnou část její taxonomické diverzity: zahrnuje 36 ze 49 známých rodů a více než 250 druhů. Výsledky fylogenetických analýz odhalily několik polyfyletických druhů i rodů a zároveň identifikovaly několik dosud nepopsaných druhů. V rámci čeďeli bylo identifikováno šest hlavních linií. Výsledky rekonstrukce biogeografické historie poukázaly na výrazný vliv lokálních i globálních tektonických, geologických, geografických a klimatických událostí na evoluční historii čeďeli Nemacheilidae.

Trojice z pěti již publikovaných článků se zaměřuje na rekonstrukci fylogenetických vztahů v rámci vybraných druhových komplexů v západní části jihovýchodní Asie. První z těchto publikací byla věnována druhovému komplexu kolem druhu *Paracanthocobitis zonalternans* a zkoumala jeho vnitřní fylogenetické vztahy a biogeografickou historii. Fylogenetické analýzy odhalily několik izolovaných linií s více méně alopatrickým rozšířením, pouze s několika případy sympatrie. Zároveň byl odhalen jeden případ potenciální hybridizace mezi dvěma liniemi. Datování odhalilo výrazný vliv výkyvů hladiny světového oceánu na evoluční historii tohoto druhového komplexu.

Druhá publikace byla zaměřena na druhový komplex *Schistura robertsi/cincticauda*, skupinu druhů obývajících menší lesní potůčky, z nichž v suchých obdobích zbývá jen několik izolovaných kaluží. Výsledky fylogenetických analýz odhalily deset jasně vymezených a geneticky izolovaných linií, z nichž pět odpovídalo již známým druhům a dalších pět představovalo druhy dosud nepopsané. I přes společný výskyt některých linií na několika lokalitách nebyly odhaleny žádné známky hybridizace a jednotlivé linie byly po bližším zkoumání spolehlivě odlišitelné na základě morfologických znaků.

Třetí publikace se zabývala skupinou druhů sdílejících podobné a unikátní zbarvení, jehož původ se předpokládá v jednom společném předkovi. Fylogenetické analýzy nicméně odhalily dvě

oddělené skupiny druhů. Kromě druhů s již zmíněným specifickým zbarvením bylo v rámci obou linií nalezeno několik druhů s úplně jiným zbarvením, a navíc i několik zástupců jiných rodů.

Čtvrtá publikace navazuje na předchozí výzkum druhového komplexu *P. zonalternans* a přináší popis nového druhu z tohoto komplexu. Tento geneticky izolovaný druh se vyznačuje unikátními morfologickými znaky a sdílí areál rozšíření s dvěma dalšími liniemi bez známek hybridizace.

Pátá publikace obsahuje popis pěti nových druhů z druhového komplexu *S. robertsi/cincticauda*. Tato práce navazuje na předchozí fylogenetickou publikaci a poskytuje praktický klíč k určení všech jedenácti nyní známých druhů tohoto druhového komplexu.

Klíčová slova: Cypriniformes, Cobitoidea, Nemacheilidae, fylogenetika, biogeografie, taxonomie, evoluční historie, *Schistura*, *Paracanthocobitis*.

Publications included in the dissertation

- I. Šlechtová, V., **Dvořák, T.**, Freyhof, J., Kottelat, M., Levin, B., Golubtsov, A., Šlechta, V., Bohlen, J. 2024. Reconstructing the evolutionary history of nemacheilid fishes across Eurasia since early Eocene. eLife, accepted with minor revisions, preprint on bioRxiv DOI: 10.1101/2024.07.05.602185
- II. Bohlen, J., **Dvořák, T.**, Šlechta, V., Šlechtová, V. 2020. Sea water shaping the freshwater biota: Hidden diversity and biogeographic history in the *Paracanthocobitis zonalternans* species complex (Teleostei: Nemacheilidae) in western Southeast Asia. Molecular Phylogenetics and Evolution. 148:106806 DOI: 10.1016/j.ympev.2020.106806. (IF 2020: 4.286).
- III. Bohlen, J., **Dvořák, T.**, Šlechta, V., Šlechtová, V. 2020. Resolving an unnoticed diversity within the *Schistura robertsi* species complex (Teleostei: Nemacheilidae) using molecules and morphology. Molecular Phylogenetics and Evolution 151:106894 DOI: 10.1016/j.ympev.2020.106894. (IF 2020: 4.286).
- IV. **Dvořák T.**, Šlechtová V., Bohlen J. 2022. Using species groups to approach the large and taxonomically unresolved freshwater fish family Nemacheilidae (Teleostei: Cypriniformes). Biology, 11(2), 175; <https://doi.org/10.3390/biology11020175>. (IF 2022: 4.2).
- V. **Dvořák, T.**, Bohlen, J., Kottelat, M., Šlechtová, V. 2023. Revision of the *Schistura cincticauda* species group (Teleostei, Nemacheilidae) using molecular and morphological markers. Scientific reports. DOI: 10.1038/s41598-023-42852-1. (IF 2023: 4.996).
- VI. **Dvořák, T.**, Šlechtová, V., Kottelat, M., Bohlen, J. 2022. *Paracanthocobitis epimekes*, a new loach species, from Myanmar and Thailand (Cypriniformes: Nemacheilidae). Ichthyological Exploration of Freshwaters. DOI: <http://doi.org/10.23788/IEF-1081>. (IF 2020: 1.682).

Other publications (not included)

- I. Bohlen, J., **Dvořák, T.** Thang, H. N. Šlechtová V. 2019. *Tanichthys kuehnei*, new species, from Central Vietnam (Cypriniformes: Cyprinidae). Ichthyological Exploration of Freshwaters. DOI: <http://doi.org/10.23788/IEF-1081>. (IF 2018/9: 0.872).
- II. Štundlová, J., Hospodářská, M., Lukšíková, K., **Dvořák, T.**, Nguyen, P., Sember, A. 2022. Sex chromosome differentiation via changes in the Y chromosome repeat landscape in African annual killifishes *Nothobranchius furzeri* and *N. kadleci*. Chromosome Research. DOI: 10.1007/s10577-022-09707-3. (IF 2022: 2.6).

Introduction

Stone loaches of the family Nemacheilidae

Distribution: The family Nemacheilidae (Eng.: 'stone loaches') is with more than 800 species one of the biggest families of freshwater fishes (Kottelat, 2012a; Nelson et al., 2016; Eschmeyer, 2024). Members of the family Nemacheilidae are widely distributed across whole Eurasia, excluding few marginal areas (Bănărescu, 1990). The distribution area of Nemacheilidae stretches from central Spain to Japan in west-east direction and from northern Russia to Java in north-south direction. Two species (genus *Afronemacheilus*) are even found in northeast Africa (Bănărescu, 1990). The distribution area of Nemacheilidae is depicted in FIG. 1. In Europe, only two genera are present, *Oxyonemacheilus* represented by three species in the Balkan Peninsula, and *Barbatula*, which is widespread with several species across the continent. One species, *Barbatula barbatula*, is also found in the Czech Republic. Besides having an enormous distribution area, Nemacheilidae are very common within this area: there is hardly any stream or river in Eurasia that is not inhabited by at least one species of stone loaches (Bănărescu, 1992). The wide distribution and common occurrence make Nemacheilidae one of the most diagnostic freshwater fish groups of Eurasia.

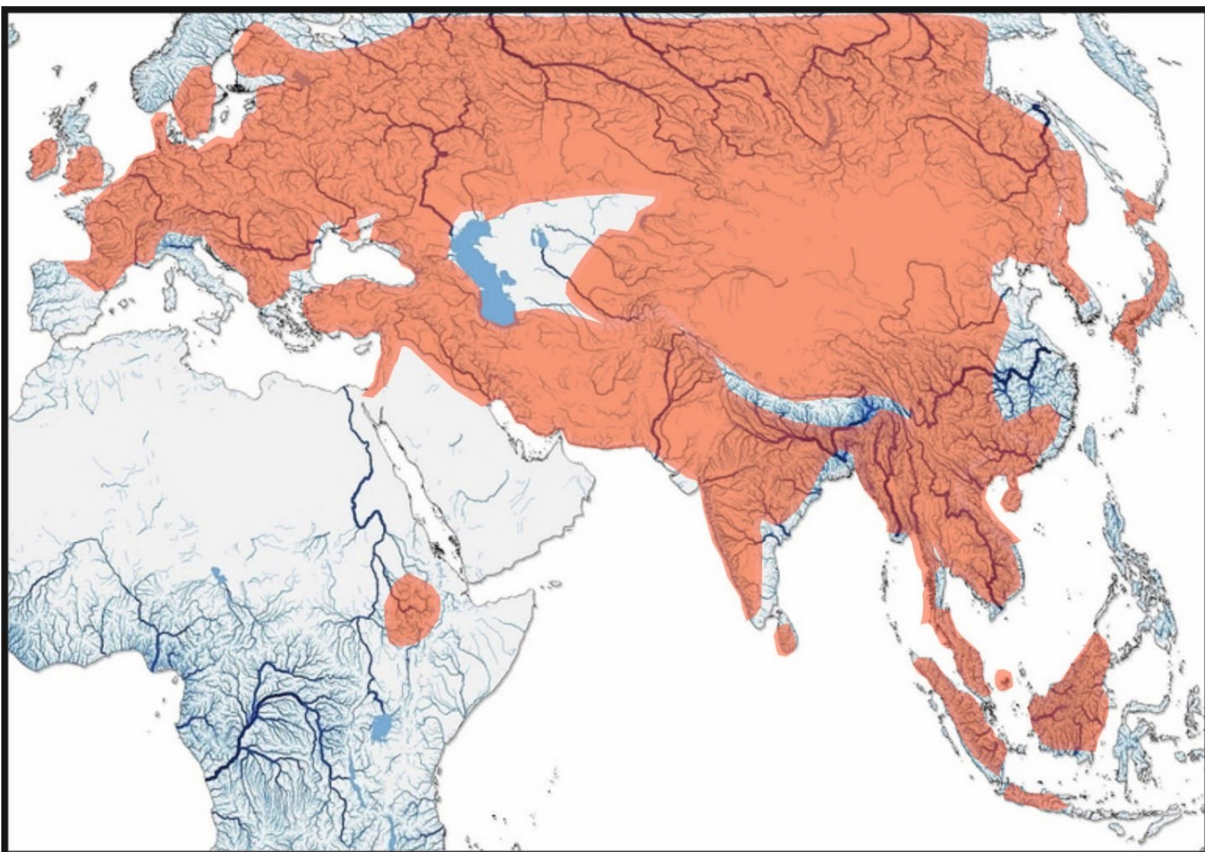


FIG. 1. Map of Eurasia and northern Africa with the distribution range of Nemacheilidae indicated by red colour.

Ecology: Fishes from the family Nemacheilidae typically occur in small to medium streams, but in general, they inhabit all available types of freshwater habitats: from small streams to lakes, swamps, caves, and even the largest rivers such as Mekong or Ganges. The ecological plasticity of Nemacheilidae is exemplified by the fact that the highest-dwelling freshwater fish species on earth (*Hedinichthys yarkandensis*, up to 5876 m a.s.l. (Day, 1877)) as well as the known deepest-dwelling cave fish (*Triplophysa gejiuensis*, more than 400 m below surface (Chu & Chen, 1979) both belong to Nemacheilidae. Their ability to colonise such diverse habitats has also significantly impacted their morphology. Nemacheilid loaches are usually small, benthic fishes around 10 cm total length; however, the family includes also many dwarf (e.g. *Physoschistura mango* (max size 2.5 cm SL; Conway & Kottelat, 2023), *Tuberoschistura cambodgiensis* (max size around 3 cm SL; Kottelat, 1990) or *Schistura diminuta* (max size 2 cm SL; Ou et al., 2011)) as well as relatively large (e.g. *Acanthocobitis pavonacea*, max size 16 cm SL (Kottelat & Vishwanath 2021)). The largest nemacheilid loach is *Triplophysa siluroides* (max size 50 cm SL and 1.5 kg; Chen et al., 2014) (see FIG. 2). Typically, nemacheilid loaches have an elongated body, although there are species with strongly elongated (e.g. *Homatula variegata*, *Homatula laxiclathra* or *Aborichthys elongatus*) or stout (*Petruichthys brevis*, *Eonemachilus nigromaculatus*) body shapes.

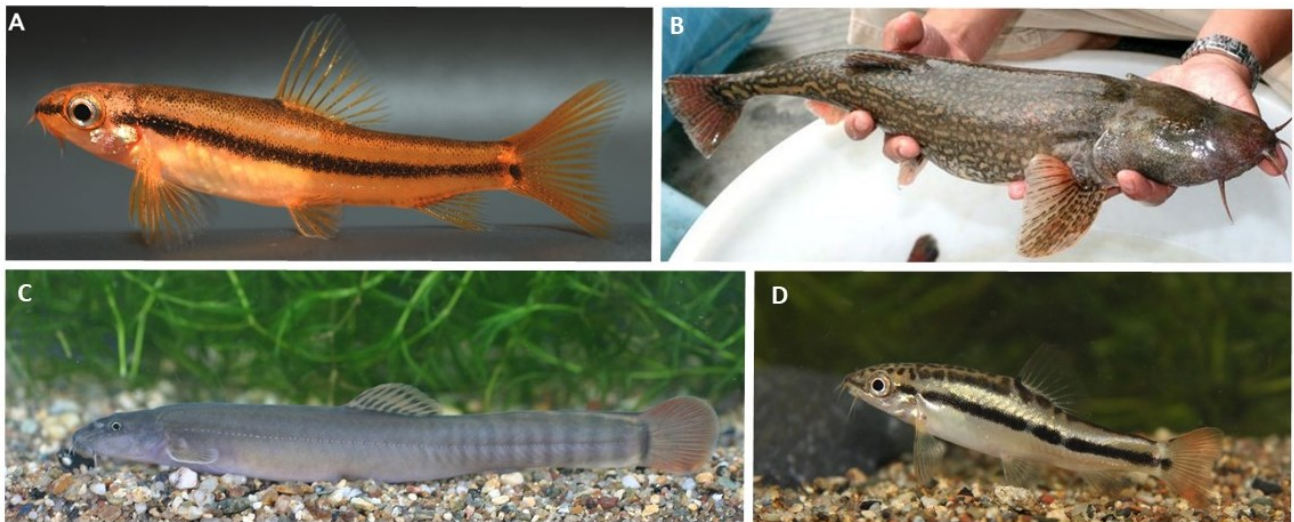


FIG. 2. Pictures of selected members of Nemacheilidae. A) *Physoschistura mango*, 22 mm SL (photo from Conway & Kottelat, 2023); B) *Triplophysa siluroides*, about 50 cm SL (photo from internet); C) *Homatula variegata*, about 14 cm SL (photo J. Bohlen); D) *Petruichthys brevis*, about 28 mm SL (photo J. Bohlen).

The mouth of most species is adapted to ubiquitous feeding on small invertebrates and organic matter, but some became scrapers that feed on algae with broad edged horny jaws (two species of the genus *Sectoria*) (Kottelat, 1990). The mouth shapes and moth structures have been traditionally considered as important diagnostic character in the nemacheilid taxonomy (FIG. 3).

Colouration: The most common pigmentation pattern in Nemacheilidae might be dark grey bands on the body on a lighter background, although the number, thickness, contrast and regularity of the bars can vary from few distinct bars to a high number of dull, worm-shaped ones. Besides, also lateral stripes, blotches or a combination of these elements are found (see FIG. 2). In the study of Dvořák et al., 2022a, a new species-group within Nemacheilidae was identified by its specific pigmentation pattern consisting of numerous thin bars on the anterior half of the body and few broad bars on the posterior half ('poculi pattern', FIG. 4). In several cave species the pigmentation pattern is nearly or completely reduced (e.g. *Draconectes narinosus* (Kottelat, 2012b), *Eidinemacheilus smithi* (Segherloo et al., 2016) or *Kayahschistura lokalayensis* (Kottelat & Grego, 2020)).

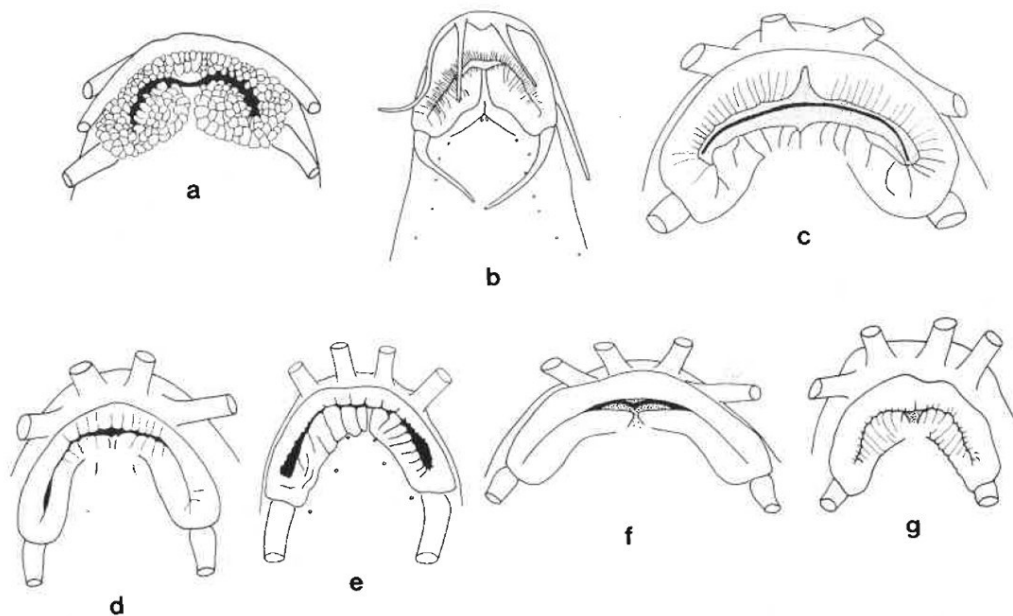


FIG. 3. Picture of mouth structures of selected nemacheilid genera. The mouth structures are traditionally considered as one of the most important diagnostic characters of external morphology in nemacheilid taxonomy: **a** *Acanthocobitis*, **b** *Neonoemacheilus*, **c** *Sectoria*, **d** *Nemacheilus binotatus*, **e** *Nemacheilus selangoricus*, **f** *Schistura*, **g** *Physoschistura*. The figure is taken from Kottelat, 1990.

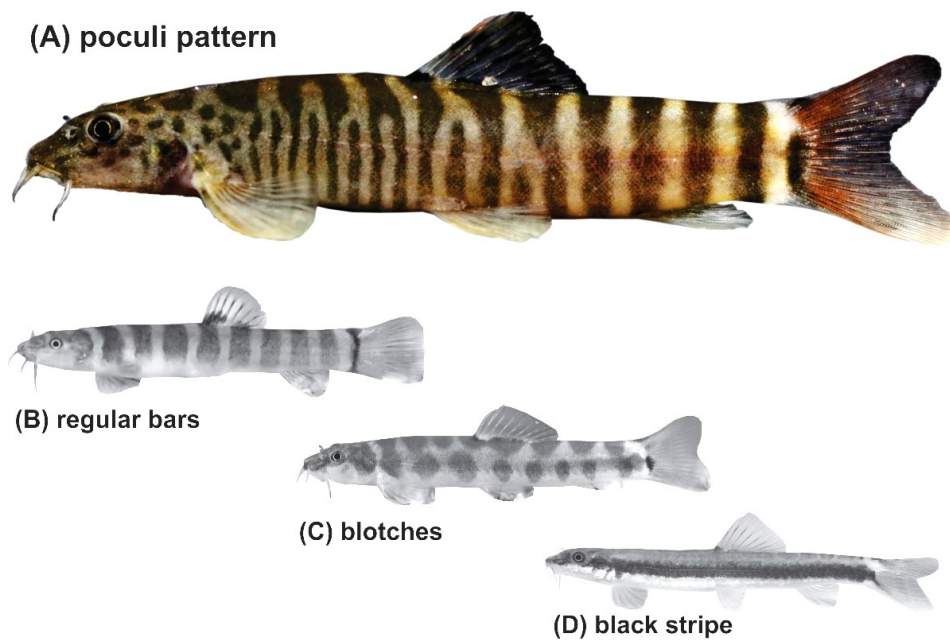


FIG. 4. Examples of different pigmentation patterns among Nemacheilidae. A) *Schistura mahnerti*, B) *Schistura robertsi*, C) *Schistura corica*, D) *Schistura pawensis* (Figure from Dvořák et al., 2022a)

Sexual dimorphism: A number of nemacheilid species develop distinct sexual dimorphism. This might in the simplest way be expressed as a smaller size and more slender body of the male, but often also with the presence of specific morphologic structures in one of the sexes; most commonly in the male. Such structure can be tubercles on head and pectoral fins (e.g. in *Barbatula* and *Triplophysa* (Šedivá, 2001; Prokofiev, 2009; Feng et al., 2020)), thickened pectoral-fin rays (e.g. *Nemacheilus* (Bohlen et al., 2022) or even large skinfolds on the pectoral fin (e.g. *Pteronemacheilus*, see FIG. 5 (Bohlen & Šlechtová, 2011)). In several genera adult males bear a suborbital flap below the eye, a lateral process of the lateral ethmoid bone (e.g. *Nemacheilus*, *Neonoemacheilus*, *Paracanthocobitis* (see FIG. 5), *Physoschistura*, *Schistura* and others (Kottelat, 1990; Bohlen & Šlechtová, 2011; Plongsesthee et al., 2012; Khyriam & Sen, 2014; Singer et al., 2017; Bohlen et al., 2022)). Different colours of both sexes are known from e.g. *Physoschistura mango* (see FIG. 5, Conway & Kottelat, 2023).

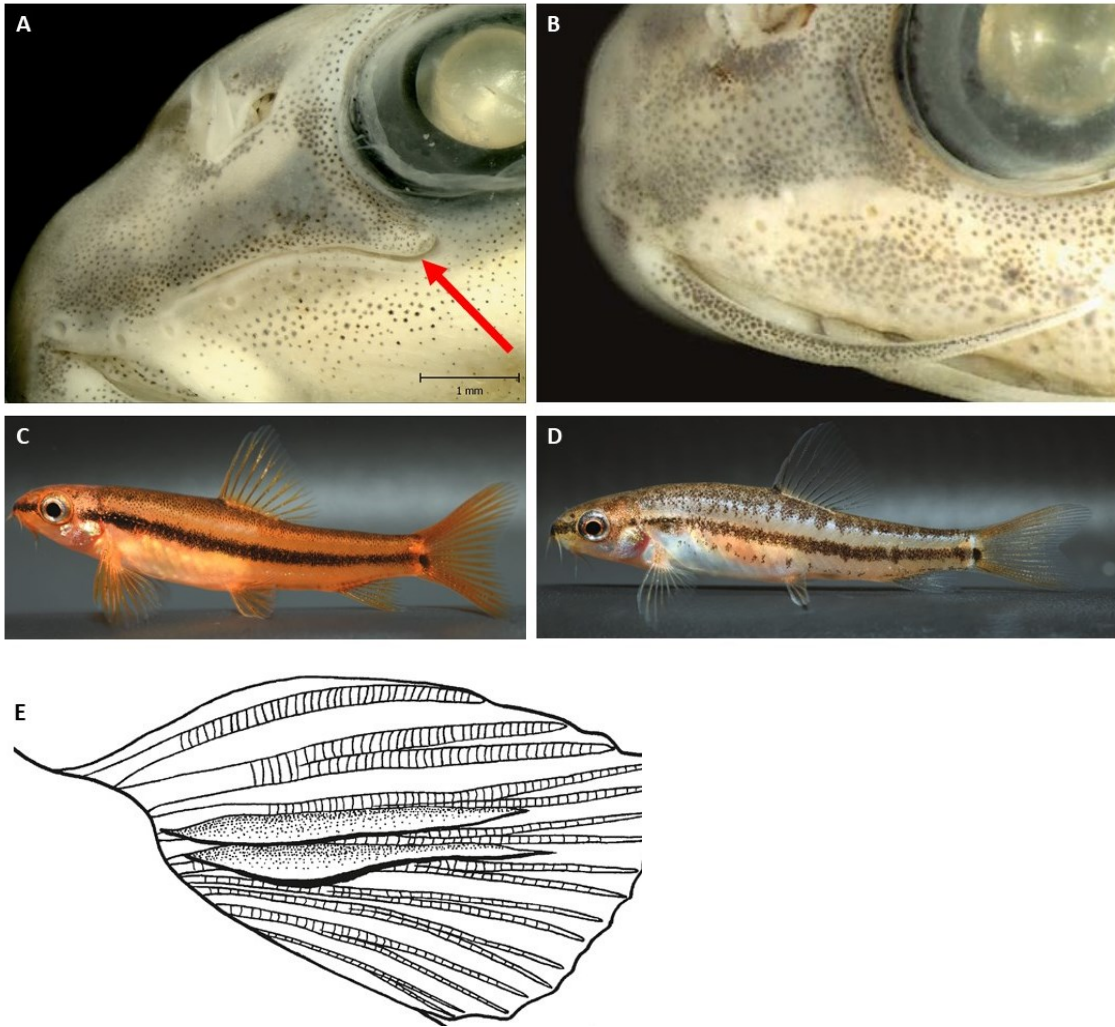


FIG. 5. Selected types of sexual dimorphism in Nemacheilidae. A) Head of male of *Paracanthocobitis epimekes*, bearing a suborbital flap under the left eye, missing in (B) female *P. epimekes*; C) male and D) female of *Physoschistura mango*; the male with a contrasting orange-black colour pattern E) right pectoral fin of male *Pteronemacheilus lucidorsum* with elongated skinfold on dorsal side of branched rays, missing in females. Figures A, B from Dvořák et al., 2022b; C, D from Conway & Kottelat, 2023; E from Bohlen & Šlechtová, 2011.

Taxonomy and classification history of Nemacheilidae

The taxonomic history of nemacheilid loaches began in 1758 with the description of the European stone loach *Cobitis barbatula* by Linnaeus (1758). Two years later Linck (1790) separated this species from the genus *Cobitis* and erected the first nemacheilid genus, *Barbatula* Linck, 1790.

Recorded observations of nemacheilid loaches outside Europe began in the early 19th century, starting in colonial India-Burma. The first of these species, *Schistura cincticauda*, *Schistura savona* and *Schistura corica*, were originally still classified under the generic name “*Cobitis*” (Hamilton, 1822). During the 19th century several new generic names appeared e.g. *Schistura* McClelland, 1838; *Acanthocobitis* Peters, 1861 and *Nemacheilus* Bleeker, 1863. In those times, taxonomic activity remained focused on India, with numerous new species descriptions being published (e.g. *Paracanthocobitis mooreh* (Sykes, 1839), *Schistura notostigma* (Bleeker, 1863) or *Schistura beavani* (Günther, 1868)).

In the first half of the 19th century, descriptions of the first two nemacheilid species from Near East appeared: *Oxynoemacheilus merga* (Krynicky, 1840) and *Oxynoemacheilus panthera* (Heckel, 1843). In the second half of the century the taxonomic efforts expanded to Southeast and East Asia, including Myanmar (*Schistura cincticauda* (Blyth, 1860)), China (*Claea dabryi* (Sauvage, 1874)) or Vietnam (*Schistura spiloptera* (Valenciennes, 1846)). This pace of a few species being described annually continued until the late 20th century. However, the burst of species descriptions appeared during the last 30-40 years, with some major publications naming 14 (Freyhof & Serov, 2001), 20 (Kottelat, 1990) or even 40 of new species (Kottelat, 2000). More than 75% of the valid species were described later than 1980 (Freyhof & Serov, 2001; Erk'Alan et al., 2007; Kottelat, 1990, 1998, 2000, 2012, 2017a-d; Prokofiev 2003, 2016; Chen et al., 2019; Eschmeyer, 2024).

Originally, loaches were classified within the family Cyprinidae as subgroups Cobitidina (including nemacheilid loaches) and Homalopterina (Günther, 1868; Vaillant, 1902). The subgroups were elevated to own families by Regan (1911), with Cobitidae divided into two subfamilies – Cobitidinae and Nemachilinae. As a subfamily of Cobitidae, stone loaches were still considered for long time by many authors (Hora, 1932, 1950; Berg, 1940 or Roberts, 1972). On the base of detailed osteological analyses (altogether 52 osteological characters), Sawada (1982) moved stone loaches from Cobitidae, which he reserved for ‘spined loaches’, into a newly established family Homalopteridae (‘hillstream loaches’) as the subfamily Noemacheilinae. Cobitidae and Homalopteridae were considered as sister families within the superfamily Cobitoidea. Moreover,

Sawada was the first author to apply Hennigian cladistics approach and propose a phylogenetic hypothesis of the superfamily Cobitoidea as well as phylogeny of all subfamilies, including Noemacheilinae. According to Kottelat (1988a), Homalopteridae was a younger synonym of Balitoridae. In the PhD thesis of Siebert (1987), the stone loaches were included in the family Balitoridae. Roberts (1989) again considered Nemacheilinae as a subfamily of Cobitidae. Nalbant & Bianco (1998) and Nalbant (2002) were the first to treat stone loaches as separate family N(o)emacheilidae, but Kottelat (2004) disagreed with the idea of Nalbant (2002) and pointed on a weak discussion and explanation of the characters used to justify the elevation of the lineages into family level. However, the position of stone loaches within Cobitoidea remained a subject of scientific debate for a long time.

The very first phylogenetic study that used molecular genetic markers and included some members of Nemacheilidae was published by Liu et al. (2004). While they utilized sequences of mitochondrial 12S rRNA, the limited number of samples included in the study restricted its ability to draw decisive conclusions.

Further studies aiming to untangle the relationships within Cobitoidea using molecular genetic markers emerged in 2005 and 2006. Saitoh et al. (2006) based their study on whole mitogenome, but with limited species coverage. Despite this, they recovered five lineages within Cobitoidea, with nemacheilins as the sister clade to balitorins. They treated the recovered clades as subfamilies, because their phylogeny remained unresolved due to weak statistical supports for the basal splits. The inferred lineages were classified as 'subfamilies of loaches', although the authors state clearly that Cobitidae and Nemacheilidae have been considered distinct families already by Nalbant (2002).

Tang et al. (2005) analysed mitochondrial cytochrome *b* (*cyt b*) mostly covering only the subfamily Botiinae with limited samples from other cobitoid subfamilies. Their analyses supported Nalbant's hypothesis (2002) to elevate the Botiinae subfamily to a family level. However, the other clades were not well covered, and authors did not comment their taxonomic level. In the following year, Tang et al. (2006) analysed mitochondrial *cyt b* and control region (CR) sequences of a wider dataset, identifying Nemacheilinae as one of six major Cobitoidea lineages. Their phylogenetic hypothesis proposed Nemacheilinae as sister clade to Cobitinae. Opposing the opinion to Saitoh et al. (2006), they supported the hypothesis of elevating the six major clades to family level.

The study of Šlechtová et al. (2007) employed the nuclear Recombination activating gene 1 (RAG 1) with the main aim to resolve the phylogeny of Cobitoidea. The study recovered the same main clades as Saitoh et al. (2006), confirmed the sister relation of nemacheiline and balitorine loaches and further suggested elevating the lineages as families within the superfamily Cobitoidea; including nemacheilines as family Nemacheilidae. Several following studies employing mitochondrial (mtDNA) sequences (Liu et al., 2010), combination of two mtDNA (Cytochrome oxidase I - COI and NADH dehydrogenase 4+5 - ND4+5) and four nuclear (nDNA) genes (Rhodopsin 1 - RH1, RAG1, Early Growth Response 2B - EGR2B and interphotoreceptor retinoid-binding protein gene 2 - IRBP2) of total length of 8541 bb (Liu et al., 2012), or combination of the whole mitochondrial genomes and four nuclear genes (RH1, RAG1, growth hormone gene – GH and IRBP2) of total length of 8541 bp (Mayden et al., 2009); and all confirmed the findings of Tang et al. (2006) and Šlechtová et al. (2007).

In the phylogenetic study by Bohlen et al. (2009), first time ever the genus *Ellopostoma* was included in a molecular phylogeny, which emerged as a new independent lineage within Cobitoidea, the family Ellopostomatidae. However, analyses based solely on nuclear RAG1 proved insufficient to resolve the polytomy among Nemacheilidae, Balitoridae and Ellopostomatidae. Subsequently, Chen et al. (2009) employed a more comprehensive dataset including six nuclear genes of 5733 bp in total length. Their findings suggested that Nemacheilidae form a sister clade to Ellopostomatidae, and together sister group to Balitoridae, represented hereby by following branching pattern: ((Balitoridae (Nemacheilidae, Ellopostomatidae)).

Kottelat (2012) elaborated a systematic revision of loach taxonomy integrating morphology with insights from previous phylogenetic studies. He accepted the family status of all main loach lineages and further divided Balitoridae into four families. However, due to ongoing debate regarding the phylogenetic position of Gyrinocheilidae and Catostomidae within the superfamily Cobitoidea as well as within the whole Cypriniformes (Šlechtová et al. 2007, 2009; Bohlen et al., 2009; Mayden et al., 2009; Chen et al., 2009; Liu et al., 2010, 2012), Kottelat restricted his focus to the suborder Cobitoidei, recognising nine families within.

Since then, the status of Nemacheilidae as a separate family has been broadly accepted. The family Nemacheilidae belongs together with further 8 families to the suborder Cobitoidea and together with families from suborder Cyprinoidea, they form the order Cypriniformes. TABLE 1 provides a comprehensive overview of taxonomical concepts associated with the superfamily

Cobitoidea and affiliation of stone loaches within. FIG. 6 depicts the currently accepted phylogeny of Cobitoidei and the position of nemacheilid loaches within the suborder.

Günther, 1868	Vaillant, 1902	Regan, 1911	Hora, 1932	Berg, 1940
Cyprinidae Homalopterina	Cyprinidae Homalopterina	Catostomidae Homalopteridae	Cobitidae Cobitinae	Cobitidae Cobitinae
Cobitidina 2 nemacheilid genera 1 botiid genus 6 cobitid genera	Cobitidina 5 nemacheilid genera 3 botiid genera 11 cobitid genera 1 ellopostomatid genus	Cobitidae Cobitidinae Nemachilinae	Nemacheilinae Homalopteridae Homalopterinae Gastromyzoninae	Botiinae Nemacheilinae
Hora, 1950	Ramaswami *	Sawada, 1982	Roberts, 1989	Nalbant, 2002 +
Cobitidae Cobitinae Nemacheilinae	Cobitidae Cobitini Botini	Cobitidae Cobitinae Botiinae	Cobitidae Cobitinae Botiinae	Botiidae Botiinae Vaillantellinae
Homalopteridae Gastromyzonidae	Nemachilini Homalopteridae Gastromyzonidae Gyrinocheilidae Psylorhynchidae Catostomidae	Homalopteridae Homalopterinae Noemacheilinae	Nemacheilinae Vaillantellinae Homalopteridae Homalopterinae Gastromyzoninae	Noemacheilidae Cobitidae
Tang et al., 2006	Nelson, 2006	Saitoh et al., 2006	Šlechtová et al., 2007	Mayden et al., 2008
Catostomidae Gyrinocheilidae Botiidae Balitoridae Gastromyzoninae Balitorinae	Catostomidae Gyrinocheilidae Cobitidae Cobitinae Botiinae Balitoridae Balitorinae	Catostomidae Gyrinocheilidae Cobitidae Cobitinae Botiinae Balitoridae Balitorinae	Catostomidae Gyrinocheilidae Vaillantellidae Botiidae Cobitidae Balitoridae Nemacheilidae	Catostomidae Gyrinocheilidae Vaillantellidae Botiidae Cobitidae Balitoridae
Cobitidae Nemacheilidae	Nemacheilinae	Nemacheilinae		Balitoridae
Bohlen et al., 2009	Chen et al., 2009	Liu et al., 2012	Kottelat, 2012a	Nelson et al., 2016
Catostomidae Gyrinocheilidae Vaillantellidae Botiidae Cobitidae Balitoridae Ellopostomatidae Nemacheilidae	Catostomidae Gyrinocheilidae Vaillantellidae Botiidae Cobitidae Balitoridae Ellopostomatidae Nemacheilidae	Botiidae Vaillantellidae Cobitidae Balitoridae Nemacheilidae	Gyrinocheilidae Vaillantellidae Botiidae Cobitidae Ellopostomatidae Nemacheilidae Barbuccidae Balitoridae Serpenticobitidae Gastromyzontidae	Catostomidae Gyrinocheilidae Vaillantellidae Botiidae Cobitidae Ellopostomatidae Nemacheilidae Barbuccidae Balitoridae Serpenticobitidae Gastromyzontidae

TABLE 1. All hypotheses on internal sorting of the superfamily Cobitoidea (modified from Dvořák, 2016). * = multiple studies (1952a, 1952b, 1952c, 1953, 1957); + = the authors did not examine representatives of all extant families. While Saitoh et al. (2006) achieved similar results to Tang et al. (2006), they did not attempt to draw any conclusions from their findings. The names of lineages with family rank are indicated in **bold**, subfamilies non-**bold**. The family or subfamily that contains Nemacheilidae is in **red letters**.

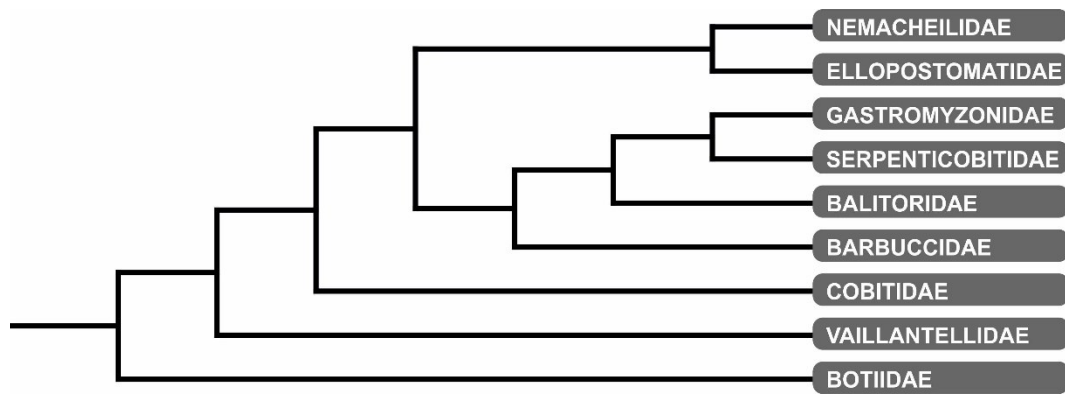


FIG. 6. The phylogenetic hypothesis of suborder Cobitoidei following Šlechtová et al. (2007, 2009), Chen et al. (2009) and Kottelat (2012).

Historical Development of Classification and Phylogeny within Nemacheilidae

The family Nemacheilidae (Cypriniformes: Cobitoidea) is the largest family of Cobitoidea, currently comprising 49 valid genera with 835 valid species according to online Eschmeyer's Catalog of Fishes (<https://www.calacademy.org/scientists/projects/catalog-of-fishes>). Traditionally, the internal classification of Nemacheilidae relied on external morphology, rarely osteology; primarily sorting similar species together into certain genera. The sorting concept usually ended with noting similarities between certain genera rather than in phylogenetic concepts for the whole family. Some of the most active ichthyologists in the creation of nemacheilid genera were/are P. Bănărescu, J. Freyhof, M. Kottelat, T.T. Nalbant, A.M. Prokofiev and H. Rendahl, which together were involved in erecting 29 of the presently valid genera. The dedicated work of these and many other ichthyologists in descriptions of species and sorting into genera provided a constantly growing and stabilising platform of knowledge about the diversity of Nemacheilidae, which is a precondition for any founded phylogenetic work with this large and taxonomically complex group.

Sawada (1982) was the first to propose a phylogenetic concept for nemacheilid loaches (considered as subfamily Noemacheilinae of family Homalopteridae in his study) based on 52 osteological characters (FIG. 7). In his study, he included 21 species of nemacheilid loaches, along with taxa from other cobitoid lineages. The included species of Nemacheilidae belong to 10 presently valid genera, and although many of these names were already available at the time of Sawada's studies, Sawada assigned his species to only two genera: *Lefua* and *Noemacheilus*. Interestingly, although the genus *Vaillantella* was not included in his comprehensive analysis, he

discussed its taxonomic affiliation and concluded, based on seven osteological characters, that it belonged to the Nemacheilidae family (Noemacheilinae in his concept).

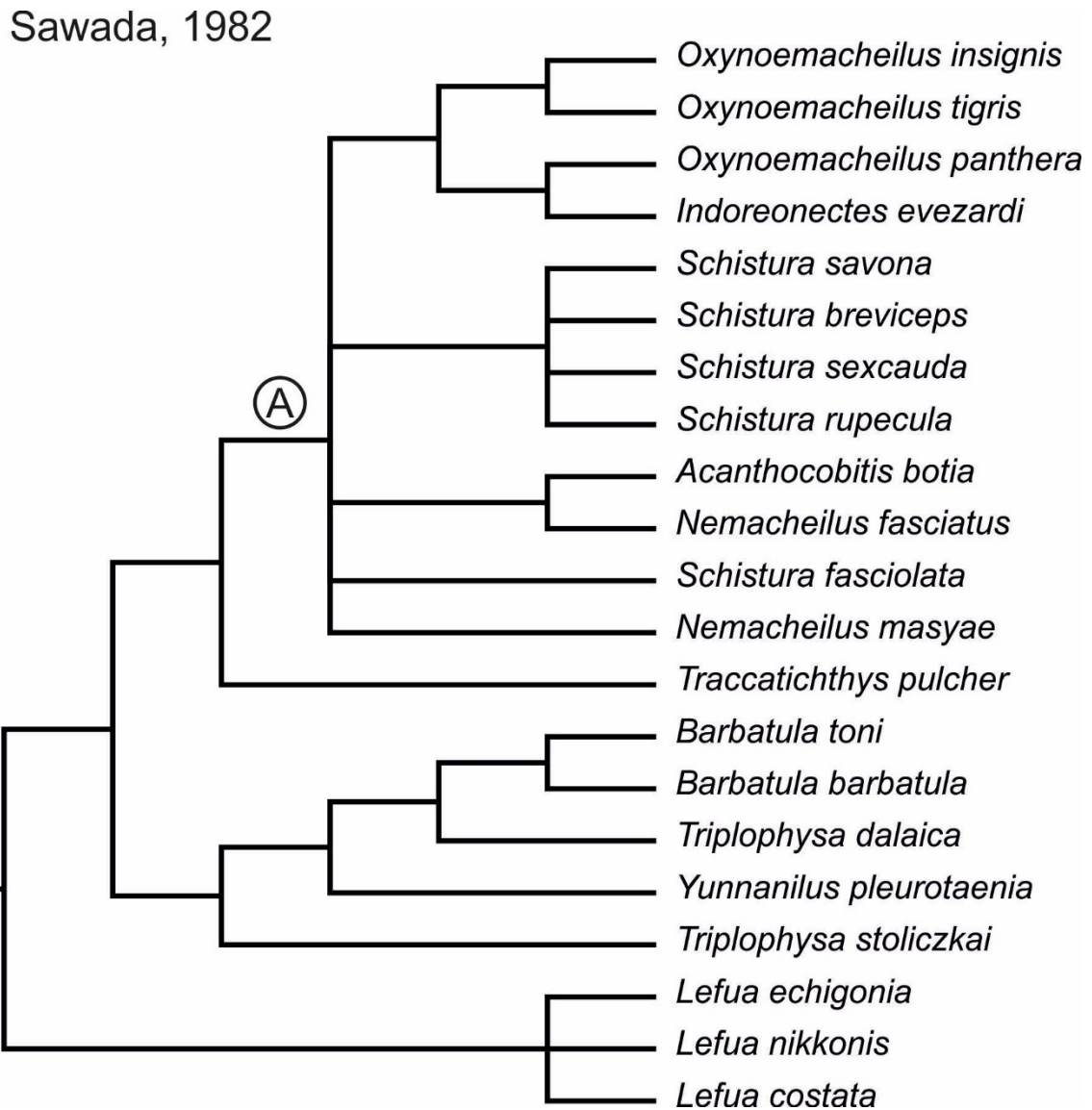


FIG. 7. Phylogeny of Nemacheilidae based on Sawada (1982) with updated nomenclature. The names of taxa in the tree reflect the current taxonomic classification of nemacheilid loaches, whereas Sawada (1982) classified all species except *Lefua* under *Noemacheilus*. Additionally, at node A (circled), Sawada's analysis generated four equally parsimonious topologies. This tree depicts the strict consensus tree derived from those topologies.

Sawada (1982) further hypothesised the biogeographical history of the superfamily Cobitoidea and each cobitoid lineage based on the branching pattern cladistically reconstructed from osteological characters. He identified South China as the centre of origin of nemacheilid loaches with subsequent dispersal in two directions: one towards Siberia and Northeast Asia,

followed by expansion to High Asia (Tibetan plateau) and Europe, and the other towards west Asia through Southeast and South Asia.

Another author who proposed a phylogenetic concept of Nemacheilidae based on comprehensive comparative analysis of morphological data was Prokofiev (2010). His phylogeny is the summary of several previous studies on the comparative morphology of nemacheilid fishes (e.g. Prokofiev, 2004, 2009). Prokofiev (2010) employed 40 significant characters of external morphology and osteology for his analysis of relationships among nemacheilid loaches (as subfamily Nemacheilinae of the family Balitoridae in his study). Based on this comprehensive analysis, he proposed a phylogenetic hypothesis with five tribes related as follows: (Vaillantellini (Lefuini (Yunnanilini (Triphophysini, Nemacheilini))). This hypothesis is also illustrated in FIG. 8. The more detailed phylogeny of the genera included in the study of Prokofiev (2010) is illustrated in FIG. 9.

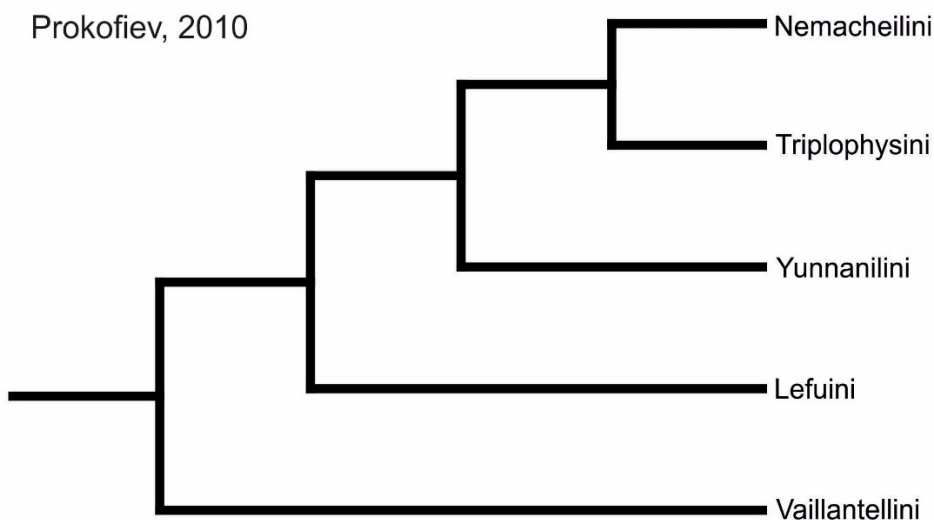


FIG 8. Relationships among nemacheilin tribes as suggested by Prokofiev 2010 on comprehensive investigation of 40 characters of osteology and external morphology. The genus *Vaillantella* (now in family Vaillantellidae) is still considered as member of Nemacheilidae.

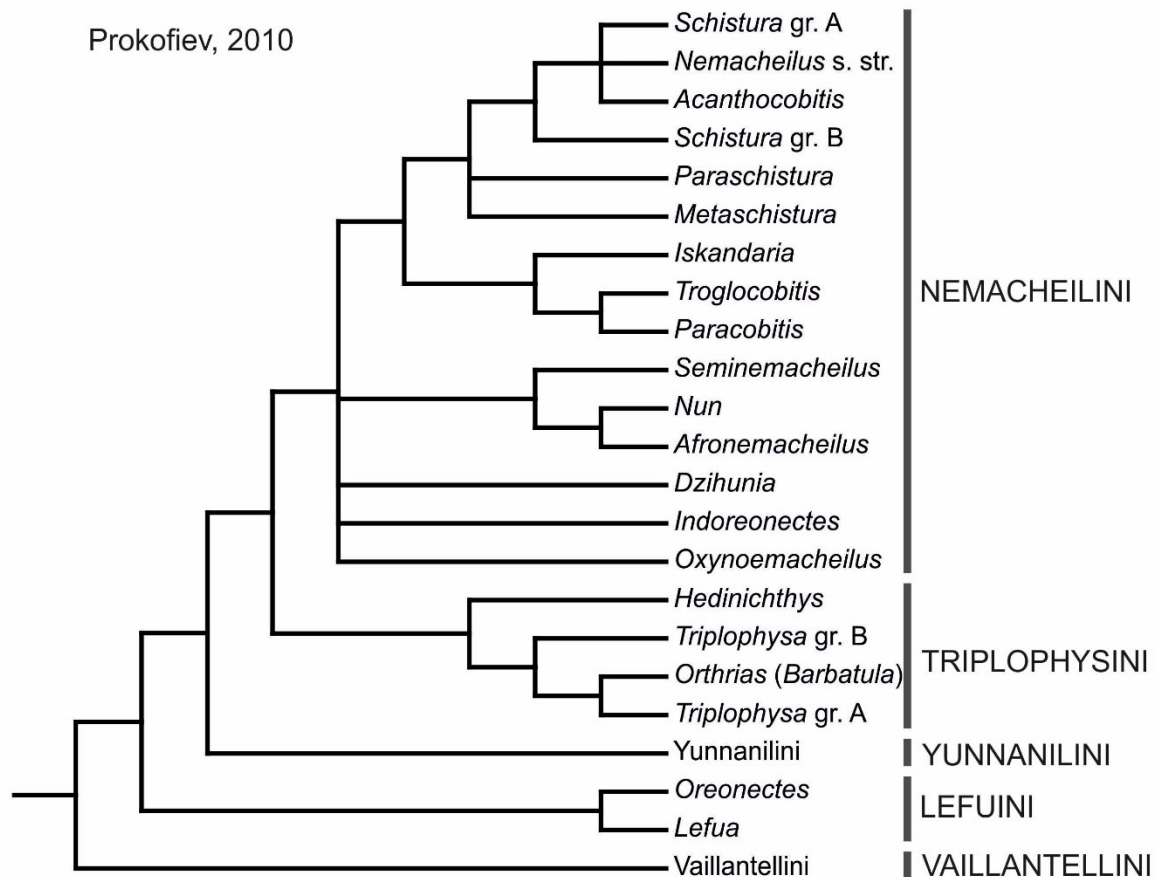


FIG. 9. Phylogenetic hypothesis of Nemacheilidae from Prokofiev 2004, 2009 and 2010 based on 40 osteological and morphological characters. Genus *Vaillantella* still considered as member of Nemacheilidae. In the original study *Barbatula* is still considered as *Orthrias*.

Building upon his analysis, Prokofiev's (2010) further proposed a classification system of Nemacheilidae assigning genera into tribes. This classification is presented in TABLE 2.

Tribe	Genus	Current status
Vaillantellini		
	<i>Vaillantella</i> Fowler, 1904	Family Vaillantellidae
Lefuini		
	<i>Lefua</i> Herzenstein, 1888	
	<i>Oreonectes</i> Günther, 1868	
	<i>Aborichthys</i> Chaudhuri, 1913	
Yunnanilini		
	<i>Yunnanilus</i> Nichols, 1925	
	<i>Eonemachilus</i> Berg, 1938	
	<i>Micronoemacheilus</i> Rendahl, 1944	
	<i>Paranemachilus</i> Zhu, 1983	
Triplophysini		

uncertain until the first molecular data based studies emerged. Saitoh et al. (2006) and Šlechtová et al. (2007) independently demonstrated that *Vaillantella* is not closely related to Nemacheilidae, and Šlechtová et al. (2007) gave first time family rank for this enigmatic genus.

Prokofiev (2010) included also *Barbucca* and *Ellopostoma* within Nemacheilinae, although the molecular phylogenies of Šlechtová et al. (2007) and Bohlen et al. (2009) had shown that none of these genera belong to Nemacheilidae but represent isolated entities within loaches (nowadays families Barbuccidae and Ellopostomatidae).

Numerous studies have employed molecular markers to investigate various aspects of Nemacheilidae. Some have focused on specific taxonomic groups, e.g. the species of *Nemacheilus* (Šlechtová et al., 2021), *Mesonoemacheilus* (Anoop et al., 2017), *Triplophysa* (Feng et al., 2019; Wang et al., 2023) or Chinese cave species in Luo et al. (2023). Other studies dealt with certain geographic regions, like Wang et al. (2016) dealt with loaches of Tibetan region. Some studies aimed to clarify the phylogenetic position of particular taxa within the broader taxonomic hierarchy (Freyhof et al., 2011, 2014, 2015, 2016, 2022). Although several studies comprised a respectable number of taxa, none of them comprehensively covered the diversity of the entire family.

Up to now, the only two studies with broader taxonomical and geographical focus were published by Chen et al. (2019) and Min et al. (2023).

Chen et al. (2019) analysed whole mitogenomes of 58 nemacheilid species belonging to 16 genera (using two novel mitogenomes and 56 published ones), nuclear RAG1 sequences of 57 species of 18 genera (two novel and 55 from GenBank) and mitochondrial COI sequences of 150 species across 26 genera (two original and 148 downloaded from BOLD). While the COI-based phylogeny lacked robust statistical support, the mitogenome analyses revealed two major clades within Nemacheilidae with high statistical support: **Clade I** includes genera distributed in the southern parts of Asia from the Near East via the Indian subcontinent, Myanmar and southern Indochina to Sundaland, including *Nemacheilus* (s. str. in concept of Šlechtová et al., 2021), *Mesonoemacheilus* (as *Nemacheilus* in the study), *Paracanthocobitis* (as *Acanthocobitis*), *Aborichthys*, *Hedinichthys*, *Schistura*, *Oxynoemacheilus* and *Petruichthys* (as *Yunnanilus*). **Clade II** comprises genera predominantly found in the northern and eastern parts of Eurasia including most of Europe, Siberia, East Asia and Indochina, including *Lefua*, *Oreonectes*, *Micronemacheilus* (as *Yunnanilus*), *Traccatichthys* (as *Micronemacheilus*), *Triplophysa*, *Claea*, *Barbatula*, *Homatula*, *Schistura* and *Tuberoschistura*. The genus *Schistura* was (as in already several former studies, e.g.

Tang et al., 2006) paraphyletic. The divergence time of these two clades was dated to approximately 36 million years ago (MYA), coinciding with the initial uplift of Tibetan plateau according to Chen et al. (2019). The RAG1-based phylogeny did not provide any support of the Clade II. Instead, in the original tree the lineage comprising *Triplophysa*, *Claea* and *Barbatula* joined Clade I, although with low statistical support. Simplified versions of both trees are depicted in FIG 10.

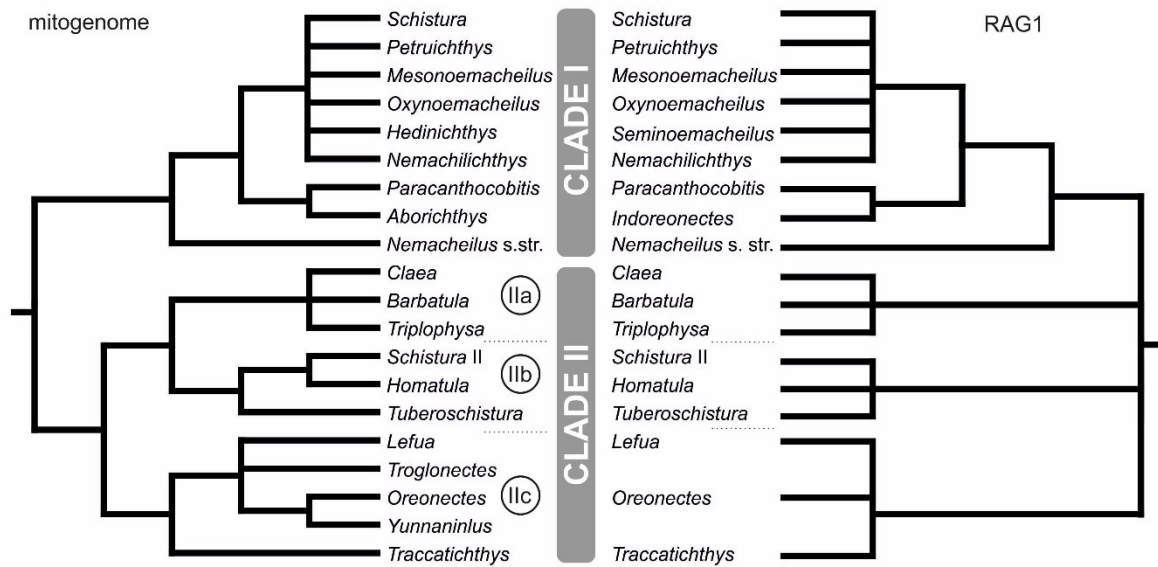


FIG. 10. Simplified cladograms based on the phylogenies presented by Chen et al. (2019). Generic names were updated to reflect current taxonomy. For ease of discussion, subclades within CLADE II have been labelled here as IIa, IIb and IIc.

Min et al. (2023) analysed a concatenated dataset of two mtDNA (*cyt b* and *COI*) and three nDNA (*RAG1*, *IRBP2* and *RH*) genes of 166 individuals representing 82 species of 18 genera. While most of the Chinese taxa were newly generated, the majority of non-Chinese sequences were obtained from GenBank. Their dated Bayesian analyses identified four major clades: a Yunnanilini-Lefuini clade, a Nemacheilini I clade, a Triplophysini clade and a Nemacheilini II clade. Their divergence time estimation dated the origin of Nemacheilidae to approximately 49.5 MYA and their biogeographic analyses (employing DIVALIKE model) identified the basins of Yangtze and Pearl River as most likely ancestral range of all Nemacheilidae. As significant driving force of diversification within Nemacheilidae they assumed the uplift of Hengduan Mountains in the southeast Himalayan arc. The nemacheilid phylogeny by Min et al. (2023) is summarised in FIG. 11.

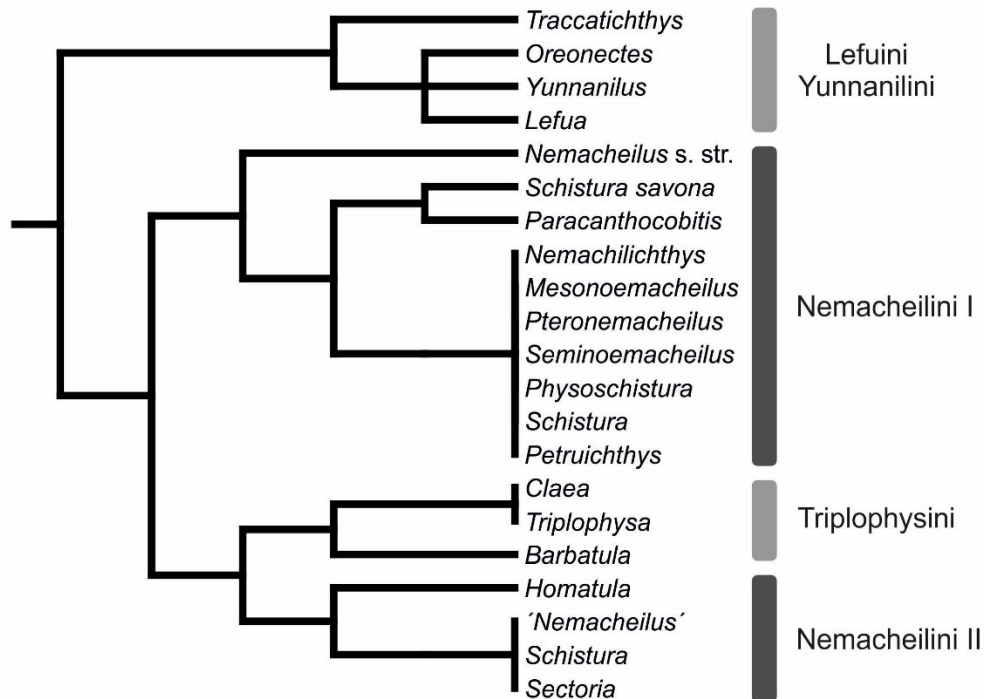


FIG. 11. Simplified cladogram based on the phylogeny presented by Min et al. (2023) derived from concatenated dataset of two mtDNA and three nDNA genes.

While both molecular and morphology-based phylogenetic studies have yielded varying results, some consistent patterns emerge. For instance, multiple analyses support a close relationship between *Triplophysa* and *Barbatula* (with some studies also including *Claea* within this group). Additionally, three of the examined phylogenetic hypotheses place *Lefua* and closely related genera in a basal position within Nemacheilidae. Despite differences in taxon sampling, the CLADE I in Chen et al. (2019) corresponds to Nemacheilini I sensu Min et al. (2023). Similarly, the IIa, IIb and IIc from Chen et al. (2019) in FIG. 10 correspond to Triplophisini, Nemacheilini II and Yunnanilini-Lefuini clades, respectively, in Min et al. (2023) (FIG. 11).

The extraordinary diversity and high number of species within Nemacheilidae, together with its extensive distribution, have likely prevented publication of a comprehensive phylogeny, covering substantial portion of distribution area and a significant portion of diversity. Previous, smaller-scale phylogenetic investigations have often suffered from sampling bias, with overrepresentation of certain lineages, or narrow geographical focus, leading to inconsistent results. Furthermore, the reuse of available GenBank data in multiple studies has introduced an element of pseudoreplication. Due to the lack of such a comprehensive study, one of the primary tasks of the present thesis is to

contribute to the first large-scale phylogeny of Nemacheilidae and to uncover its biogeographic patterns and its evolutionary history.

Genera of Nemacheilidae

The family Nemacheilidae currently contains around 50 valid genera (Eschmeyer, 2024). The number is difficult to estimate exactly due to different opinions among authors about validity of certain genera. Moreover, new genus descriptions (*Eidinemacheilus* Segherloo, Ghaedrahmati & Freyhof, 2016; *Sasanidus* Freyhof, Geiger, Goltzarianpour & Patimar, 2016; *Troglonectes* Zhang, Zhao & Tang, 2016; *Malihkaia* Kottelat, 2017; *Mustura* Kottelat, 2018; *Kayahschistura* Kottelat & Grego, 2020; *Kapuasias* Kottelat & Tan, 2024), taxonomic reviews (Prokofiev, 2017; Du et al., 2023; Luo et al., 2023) and application of modern molecular genetics methods contribute to dynamics in nemacheilid classification.

The two most species-rich genera within Nemacheilidae are *Schistura* and *Triplophysa*, with 241 and 169 valid species, respectively. 14 nemacheilid genera contain more than ten species, while the rest includes less than ten species. Several genera are monotypic, containing only a single species. A full list of nemacheilid genera is provided in TABLE 3.

Genus name	Number of species	Author and year of description	Synonyms	Type species
<i>Aborichthys</i>	14	Chaudhuri 1913		<i>A. kempfi</i>
<i>Acanthocobitis</i>	2	Peters 1861		<i>A. longipinnis</i>
<i>Afronemacheilus</i>	2	Golubtsov & Prokofiev 2009		<i>A. abyssinicus</i>
<i>Barbatula</i>	29	Linck 1790	<i>Cobites</i> , <i>Orthrias</i>	<i>B. barbatula</i>
<i>Claea</i>	2	Kottelat 2011	<i>Oreias</i>	<i>C. dabryi</i>
<i>Draconectes</i>	1	Kottelat 2012		<i>D. narinosus</i>
<i>Dzihunia</i>	3	Prokofiev 2001		<i>D. amudaryensis</i>
<i>Eidinemacheilus</i>	2	Segherloo, Ghaedrahmati & Freyhof 2016		<i>E. smithi</i>
<i>Eonemachilus</i>	9	Berg 1938		<i>E. nigromaculatus</i>
<i>Hedinichthys</i>	4	Rendahl 1933	<i>Minihedinichthys</i>	<i>H. yarkandensis</i>
<i>Homatula</i>	28	Nichols 1925		<i>H. potanini</i>
<i>Indoreonectes</i>	5	Rita & Bănărescu 1978		<i>I. keralensis</i>
<i>Iskandaria</i>	2	Prokofiev 2009		<i>I. kuschakewitschi</i>
<i>Kapuasias</i>	1	Kottelat & Tan 2024		<i>K. maculiceps</i>
<i>Karstsinnectes</i>	6	Zhou, Luo, Wang, Zhou & Xiao 2023		<i>K. anophthalmus</i>
<i>Kayahschistura</i>	1	Kottelat & Grego 2020		<i>K. lokalayensis</i>
<i>Lefua</i>	7	Herzenstein 1888	<i>Elxis</i> , <i>Octonema</i>	<i>L. pleskei</i>
<i>Malihkaia</i>	1	Kottelat 2017		<i>M. aligera</i>
<i>Mesonoemacheilus</i>	10	Bănărescu & Nalbant 1982		<i>M. triangularis</i>
<i>Micronemacheilus</i>	4	Rendahl 1944		<i>M. cruciatus</i>
<i>Mustura</i>	18	Kottelat 2018		<i>M. celata</i>
<i>Nemacheilus</i>	37	Bleeker 1863	<i>Modigliania</i> , <i>Pogononemacheilus</i>	<i>N. fasciatus</i>
<i>Nemachilichthys</i>	1	Day 1878		<i>N. ruppelli</i>

<i>Neonemacheilus</i>	5	Zhu & Guo 1985	<i>Infundibulatus</i>	<i>N. labeosus</i>
<i>Nun</i>	1	Bănărescu & Nalbant 1982		<i>N. galilaeus</i>
<i>Oreonectes</i>	7	Günther 1868	<i>Octonema</i>	<i>O. platycephalus</i>
<i>Oxyonemacheilus</i>	72	Bănărescu & Nalbant 1966	<i>Ilamnemacheilus</i>	<i>O. persa</i>
<i>Paracanthocobitis</i>	21	Grant 2007		<i>P. zonalternans</i>
<i>Paracobitis</i>	13	Bleeker 1863	<i>Adiposia, Pseudodon, Troglcobitis</i>	<i>P. malapterura</i>
<i>Paranemachilus</i>	4	Zhu 1983	<i>Heminoemacheilus</i>	<i>P. genilepis</i>
<i>Paraschistura</i>	27	Prokofiev 2009	<i>Metaschistura</i>	<i>P. sargadensis</i>
<i>Petruichthys</i>	2	Menon 1987		<i>P. brevis</i>
<i>Physoschistura</i>	10	Bănărescu & Nalbant 1982		<i>P. brunneana</i>
<i>Protonemacheilus</i>	1	Yang & Chu 1990		<i>P. longipectoralis</i>
<i>Pteronemacheilus</i>	2	Bohlen & Šlechtová 2011		<i>P. lucidorsum</i>
<i>Rhyacoschistura</i>	3	Kottelat 2019		<i>R. larreci</i>
<i>Sasanidus</i>	1	Freyhof, Geiger, Golzarianpour & Patimar 2016		<i>S. kermanshahensis</i>
<i>Schistura</i>	241	McClelland 1838	<i>Acoura</i>	<i>S. rupecula</i>
<i>Sectoria</i>	2	Kottelat 1990		<i>S. atriceps</i>
<i>Seminoemacheilus</i>	6	Bănărescu & Nalbant 1995		<i>S. lendlii</i>
<i>Speonectes</i>	1	Kottelat 2012		<i>S. tiomanensis</i>
<i>Sphaerophysa</i>	1	Cao & Zhu 1988		<i>S. dianchiensis</i>
<i>Sundoreonectes</i>	3	Kottelat 1990		<i>S. obesus</i>
<i>Traccatichthys</i>	5	Freyhof & Serov 2001		<i>T. taeniatus</i>
<i>Triplophysa</i>	169	Rendahl 1933	<i>Daducobitis, Deuterophysa, Didymophysa, Diplophysa, Diplophysoides, Indotriplophysa, Labiatophysa, Paradidymophysa, Qinghaichthys, Sclerophysa, Tarimichthys, Tauphysa</i>	<i>T. hutjertjuensis</i>
<i>Troglonectes</i>	20	Zhang, Zhao & Tang 2016		<i>T. furcocaudalis</i>
<i>Tuberoschistura</i>	2	Kottelat 1990		<i>T. baenzingeri</i>
<i>Turcinoemacheilus</i>	11	Bănărescu & Nalbant 1964		<i>T. kosswigi</i>
<i>Yunnanilus</i>	19	Nichols 1925		<i>Y. pleurotaenia</i>
	838			

TABLE 3. Genera of Nemacheilidae with overview about their number of species.

Genera are colour-coded as follows: green indicates genera where the type species is used in any of the publications included in the thesis; blue indicates genera where other than the type species is/are used; yellow indicates genera not used in our studies and red indicates genera, which are extinct.

Schistura, with 241 valid species distributed across the Indian subcontinent, southern China, Myanmar and most of the Indochinese peninsula, represents the most species-rich genus within Nemacheilidae. However, this primacy is most likely temporary, since *Schistura* was considered already by Kottelat (1990) as ‘catch-all genus’ and by Bănărescu & Nalbant (1995) as ‘polyphyletic’. Also, several subsequent molecular genetic studies have shown that *Schistura* is not a monophyletic genus, but a collection of similar-looking species dispersed across the Nemacheilidae phylogeny (Tang et al., 2006; Kottelat, 2012a, 2017a-c; Sember et al., 2015; Freyhof et al., 2016; Sgouros et al., 2019). Despite this polyphyly, *Schistura* includes several well-defined species groups with distinct

morphological characters such as *Schistura cincticauda* group (Bohlen et al., 2020; Dvořák et al., 2023) or *Schistura poculi* group (Dvořák et al., 2022).

The second biggest genus is *Triplophysa* with 169 valid species. *Triplophysa* is mainly distributed in China and Mongolia, however its range extends into northern India, south-eastern Russia, eastern parts of central Asian countries and northeastern parts of Afghanistan and Pakistan. Several studies have investigated the phylogeny within *Triplophysa* (Prokofiev, 2010; Wang et al., 2016; Feng et al., 2019; Wu et al., 2020; Wang et al., 2023).

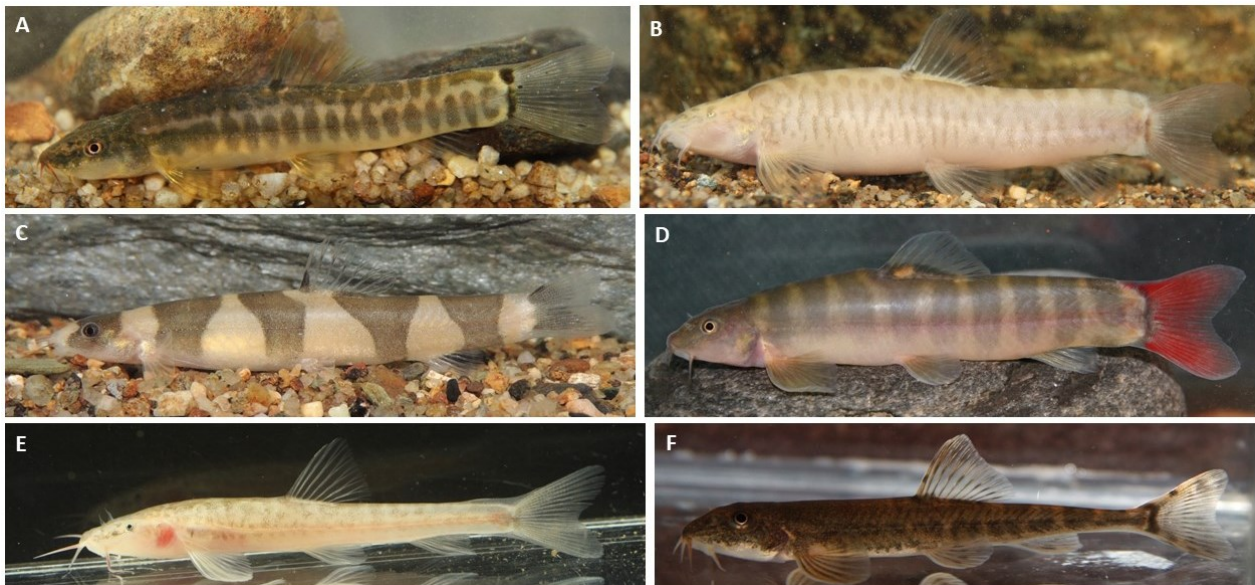


FIG.12. Pictures of selected species of *Schistura* and *Triplophysa*. A) *Schistura pantherina*; B) *Schistura jarutanini*; C) *Schistura pridii*; D) *Schistura yersini*; E) *Triplophysa luochengensis*; F) *Triplophysa stenura* (photos J. Bohlen/ V. Šlechtová).

Barbatula belongs with 29 currently recognized species among the medium-sized genera (10-100 species). Despite its wide distribution (largest among Nemacheilidae), spanning from the Iberian Peninsula eastward across most of Europe, Western Asia, Mongolia, northeast China and Siberia to the Japanese Islands (Šedivá et al., 2008; Eschmeyer, 2024), the taxonomic knowledge of *Barbatula* remains limited. Šedivá et al. (2008) identified five distinct clades within the Danube River basin alone, suggesting a significant underestimation of species diversity across the genus's range. Notably, a population *Barbatula barbatula*, inhabiting an underground lake close to Swabian Alb in southern Germany represents the only known cavefish in Europe (Behrmann-Godel et al, 2017). Another "medium species rich" genus is *Oxynoemacheilus*, comprising approximately 72 species (Freyhof et al., 2012; Eschmeyer, 2024). As one of only two nemacheilid genera occurring in Europe, it is widespread in the Near East, from eastern Iran through whole Turkey to the southern part of

the Balkan Peninsula. Despite their shared European distribution, *Oxynoemacheilus* and *Barbatula* are not closely related and exhibit distinct colonisation histories (Sember et al., 2015; Freyhof et al., 2016; Chen et al., 2019; Šlechtová et al., 2024). *Oxynoemacheilus* includes both widespread species as well as numerous endemic taxa restricted to small lakes or endorheic basins (Freyhof et al., 2012; Freyhof, 2016; Çiçek et al., 2018; Saygun et al., 2021; Seçer et al., 2022; Turan et al., 2023).

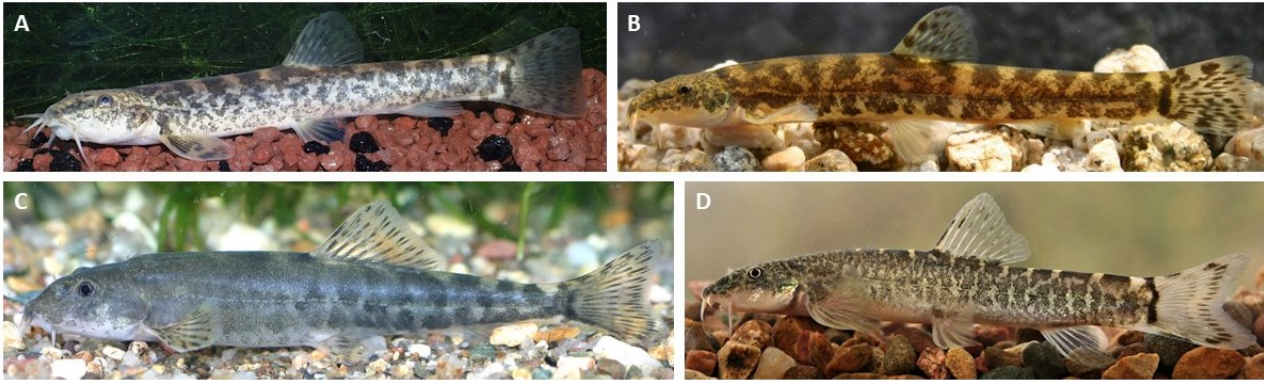


FIG.13. Pictures of selected species of *Barbatula* and *Oxynoemacheilus*. A) *Barbatula barbatula*; B) *Barbatula* indet; C) *Oxynoemacheilus* indet (photos J. Bohlen/V. Šlechtová); D) *Oxynoemacheilus pindus* (photo A. Šedivá).

Another 13 ‘medium-rich’ genera are exclusively restricted to Asia: ***Aborichthys*** is a northeast Indian element, since all its 14 valid species occur in India with only few records from neighbouring countries Myanmar, Bangladesh and Nepal. Species of *Aborichthys* are characterised by a strongly elongated body and a rounded caudal fin (Nanda & Tamang, 2021; Singh & Kosygin, 2022). Another typically Indian genus ***Mesonoemacheilus***, that is endemic to the Western Ghats, comprises 10 recognized species (Anoop et al., 2017). Due to ornamental coloration, several *Mesonoemacheilus* species are popular in the aquarium fish trade. ***Homatula***, predominantly distributed across the upper reaches of major Chinese river systems (Yangtze, Pearl, Red, Salween and Mekong River basins), includes 28 recognized species, characterized by strongly elongated bodies and rounded to truncate caudal fish base (Che et al., 2023). ***Mustura***, relatively recently described in 2018, is distinguished by the presence of a manubrium connecting the two air bladder capsules and by pectoral fin slanted upwards and with small tubercles on unculiferous pads along the anterior branched fin rays in males (Kottelat, 2018). *Mustura* contains 18 valid species, with additional taxa potentially belonging to this genus. Many species currently classified within *Mustura* were previously assigned to *Physoschistura*. (Choudhury et al., 2021; Qin et al., 2022; Eschmeyer, 2024). ***Nemacheilus***, the name bearing genus of the family, includes currently 37 valid species. Distributed across the Indochinese Peninsula, Malay Peninsula and Sundaic Islands (Borneo,

Sumatra and Java), it represents the southernmost extent of Nemacheilidae (Kottelat, 2012a; Eschmeyer, 2024). A recent phylogenetic study (Šlechtová et al., 2021) revealed that several species classified within *Nemacheilus* belong to distinct lineages. ***Paracanthocobitis*** contains 21 species with distribution that covers a continuous stripe across the north of the Indian subcontinent, Myanmar and western Thailand plus two isolated areas of occurrence; one in the lower Mekong basin in Cambodia and Laos and one in the Western Ghats in India and on Sri Lanka (Rainboth et al., 2012; Singer & Page, 2015; Kosygin et al., 2022). The distribution crosses multiple significant biogeographic barriers, which generally are known to represent shifting points between faunistic regions. Therefore, *Paracanthocobitis* is an ideal model for investigating biogeographical, geological or climatic factors shaping the biodiversity of the region (Singer et al., 2017; Bohlen et al., 2020). ***Paracobitis*** is another Near East faunistic element with 13 valid species distributed across Iran, Iraq, Afghanistan and Uzbekistan. The genus is characterised by elongated body and presence of a very deep dorsal and small ventral adipose crests on caudal peduncle (Freyhof et al., 2014). ***Paraschistura*** is another substantial genus, with 27 species, and one of the most widespread. Species of *Paraschistura* occur in rivers from eastern Turkey through Iran, Turkmenistan, Afghanistan, Pakistan and western parts of India. This genus exhibits remarkable morphological variability, ranging from stout-bodied forms like *P. cristata* to very elongated species such as *P. abdolii*. Most species share the presence of a suborbital flap in males as sexual dimorphism (Freyhof et al., 2015). As mentioned above, ***Physoschistura*** currently including 10 species, was formerly a more inclusive taxon. Numerous members have been reclassified into recently established genus *Mustura*. Similar to *Schistura*, *Physoschistura* is considered polyphyletic with unclear relationships to other genera as well as inside (Conway & Kottelat, 2024). ***Troglonectes***, the strictly Chinese genus established recently (Zhang et al., 2016), contains nowadays 20 valid species occurring in karst areas in the Guangxi and Guizhou provinces in south-eastern China. Some of them are troglomorphic endemics of single caves (Du et al., 2023; Zhao et al., 2024; Ge et al., 2024). ***Turcinoemacheilus*** is another Near East genus, although one of its 11 valid species, *T. himalaya* is known from Nepal; the others occur in high-altitude mountainous streams in Iran and Turkey, several of them as a very local endemics of one small stream. (Jouladeh-Roudbar et al., 2023). ***Yunnanilus***, another typical freshwater element of southeast China, includes currently 19 recognised species. Although once more diverse, it has undergone taxonomic revision resulting in the transfer of numerous species to

Eonemacheilus and other genera. *Yunnanilus* is predominantly distributed in upper Pearl River, with a limited presence in upper Yangtze and Red River basins (Wen et al., 2022; Du et al., 2023).



FIG.14. Pictures of selected species of ‘medium-rich’ genera. A) *Nemacheilus binotatus*; B) *Mesonoemacheilus triangularis*; C) *Aborichthys indet*; D) *Mustura shuensis* (photos J. Bohlen/V. Šlechtová); E) *Turcinoemacheilus minimus* (photo from Esmaeili et al., 2014); F) *Paracanthocobitis pictilis* (photo J. Bohlen).

Acanthocobitis belongs among the smaller nemacheilid genera (2 - 9 valid species). Although, it was once a more diverse genus, most of its species were moved to the newly erected genus *Paracanthocobitis* (Grant, 2007) and presently contains only two described and at least one undescribed species (Kottelat & Vishwanath, 2021). *Acanthocobitis* is notable for its large-bodied species and the unique possession of a pointed caudal fin within the family (Singer & Page, 2015). The only African genus of Nemacheilidae, ***Afronemacheilus***, is with two species endemic to Ethiopia. Some preliminary data based on COI sequences show that *Afronemacheilus* does not belong to the clade containing the common widespread near east genera (e.g. *Oxyonemacheilus*) but is sister to the genus *Iskandaria* from Middle Asia (Prokofiev & Golubtsov, 2013). ***Claea*** comprises two species inhabiting fast flowing rivers of southwest China with range extension into the Tibetan plateau. The genus was previously known as “*Oreias*”, but since the name was preoccupied within vertebrates, it was renamed *Claea* by (Kottelat, 2011). In numerous phylogenetic studies, *Claea* is found nested within *Triplophysa* (e.g. Li et al., 2015; Wang et al., 2016; Chen et al., 2019; Min et al., 2023). Three valid species of ***Dzihunia*** distributed in Central Asia inhabit endorheic basins, two of them in the Amudarja and Syrdarja river systems flowing into remnants of the Aral Sea and one in the Talas River basin. *Dzihunia* is characterized mainly by very long and thin caudal peduncle. *Dzihunia* can be

distinguished from morphologically similar *Triplophysa* by different habitat, mouth structure and the presence of spinule like epidermal formations on the skin (Prokofiev, 2009). Last molecular study of Uzbekistan's fishes based on CO I sequences revealed existence of three more undescribed species of *Dzhunia* (Sheraliev & Peng, 2021). ***Eidinemacheilus*** contains two troglomorphic species endemic to the Tigris River drainage. Notably, these species exhibit a disjunctive distribution, separated by over 500 km. *E. smithi* is endemic to a spring and subterranean waters near Baq-e-Loveh Oasis in Zagros Mountains in Iran. *E. proudlovei* is endemic to an ephemeral spring in the Tabeen Gorge of Pirmagrun Mountain in Iraq. The discovery of *E. proudlovei* is particularly noteworthy. After an exceptionally heavy rains, hundreds of blind, pink fishes were flushed from a temporary spring. Local reports indicated that such an event was absolutely unique, and ichthyologists have speculated about potential for undiscovered subterranean fish diversity within the arid Near East (Segherloo et al., 2016). ***Eonemachilus*** is a Chinese endemic genus comprising nine valid species, characterised by a short, deep body and a terminal mouth. *Eonemachilus* was historically considered as a synonym to *Yunnanilus*. The validity of *Eonemachilus* was generally accepted following comprehensive molecular phylogenetic studies of the Yunnanilini clade (Du et al., 2021; Du et al., 2023). ***Hedinichthys*** with its four valid species occurs in western China, with one species extending into northwest India. Species of this genus are characterised by scaleless bodies, smooth lips and exceptionally slender caudal peduncle (Prokofiev, 2010). ***Indoreonectes***, endemic to Western Ghats and Godavari River in India, contains five valid species. *Indoreonectes* is characterized by having very long barbels, long nasal tubes and a distinctive colour pattern of alternating bars and dorsal saddles (Prasad et al., 2020; Kumkar et al., 2021). ***Iskandaria*** with the only two valid species is a genus endemic for Middle Asia, namely Kazakhstan, Uzbekistan and Tadzhikistan. Species of *Iskandaria* are characterised by a comparably slender and shallow body, by small eyes and by the presence of a prominent axillary pelvic lobe (Prokofiev, 2009). ***Karstsinnectes*** is a troglomorphic genus endemic to the karst area in Guangxi and Guizhou provinces in southeast China. Species of *Karstsinnectes* have a prominent dorsal and ventral adipose crest on caudal peduncle, prolonged snout and forked or truncate caudal fin. Several species completely miss the lateral line and head sensory pores (Luo et al., 2023; Ge et al., 2024). ***Lefua***, comprising eight valid species, occurs in Japan, Korean peninsula, northeastern China and the south-eastern tip of Russia. All *Lefua* species share the presence of a rounded caudal fin. Several species of *Lefua* show a strong sexual dimorphism in colouration, with males possessing a prominent midlateral stripe absent in

females (Prokofiev, 2010). Another genus characterised by a rounded caudal fin (besides *Acanthocobitis* and *Lefua*) is ***Oreonectes*** distributed with its seven valid species in south and southeast China. *Oreonectes* and *Lefua* are morphologically very similar, share five osteological synapomorphies and differ in only three osteological characters (Prokofiev, 2005). ***Micronemacheilus*** occurs with 3 species in Southwest China and one species in central Vietnam. Species of genus *Micronemacheilus* are characterised by high body, pelagic lifestyle and by pigmentation pattern consisting of prominent dark bars on light body (Prokofiev, 2010). *Micronemacheilus* has been shown to be polyphyletic (Du et al., 2021), thus a new genus “*Guinemachilus*” was erected for the single species *M. longibarbatus* (Du et al., 2023). However, the new genus is not valid due to the absence of ZooBank registration (Eschmeyer, 2024).



FIG.15. Pictures of selected species of ‘medium-rich’ genera. A) *Afronemacheilus abyssinicus* (photo L. Kent); B) *Lefua costata*, male; C) *Lefua costata*, female; D) *Micronemacheilus cruciatus*; E) *Oreonectes* sp. (photos J. Bohlen/V. Šlechtová).

Neonoemacheilus includes five species distributed mostly in North India with some species reaching to Myanmar, northern Thailand and southern China. Species of *Neonoemacheilus* are characterised by 14 to 21 dark bars on body side, very thick and structured lips and presence of suborbital flap in males (Khyriam & Sen, 2014). ***Paranemachilus*** is an endemic genus of the Guangxi province in southern China, comprising four species. Species of genus *Paranemachilus* are characterised by pigmentation pattern consisting of small dark dots covering the whole body sides and by presence of scales on the sides of the head (Prokofiev, 2010). Like many other subterranean and semi- subterranean fishes in South China, these species face significant threats from human activities due to road construction, limestone mining, erosion, and increasing tourism (Wen et al.,

2022). ***Petruichthys*** contains presently two species. While *P. brevis*, endemic to Inle Lake (Salween River drainage) is well known and studied, *P. salmonides* in southern Yunnan is either considered as synonym of *Yunnanilus pleurotaenia* (e.g. Zhu, 1989) or mentioned to be a species of a doubtful identity requiring further investigation (*species inquirenda et incertae sedis*) (Kottelat, 2013). *P. brevis* is characterized by a stout body, comparably big eyes and pronounced sexual dimorphism (Bohlen & Šlechtová, 2011). This species is occasionally exported for the ornamental fish trade. ***Pteronemacheilus*** includes two valid species: *P. lucidorsum* from Irrawaddy drainage in North Myanmar and on *P. meridionalis* from upper Mekong in southern China. The genus is characterised by the presence of elongated skinfolds on the dorsal side of the second and third branched pectoral-fin rays in males, a distinctive character that inspired the generic name (Bohlen & Šlechtová, 2011). Recently described ***Rhyacoschistura*** includes three species, two (*R. larreci* and *R. suber*) distributed in Laos and one (*R. maejotigrina*) found in Thailand. Species of *Rhyacoschistura* are characterised by modified pectoral fins in males, with a very thick first ray and tuberculated anterior rays and/or membranes (Kottelat, 2019). ***Sectoria*** comprises two species: one in upper Chao Phraya River (Thailand) and one in middle Mekong (southwest China and northwest Laos). The genus is characterised by a specific mouth morphology adapted for scraping algae from stones (Kottelat, 1990; Chen, 1999). ***Seminemacheilus***, a Near East genus, comprises six species primarily distributed in eastern Turkey, with one exception (*S. tongiorgii*) found in Iran. *Seminemacheilus* is characterised by the absence of epural bones in the caudal skeleton, a slightly emarginate or truncate caudal fin, and elongated pectoral fins reaching behind the origin of pelvic fin in males (Freyhof et al., 2016). ***Sundoreonectes***, endemic to Borneo, includes two species inhabiting typical riverine habitat. Both species are characterised by rounded caudal fin (Chin, 1990; Bănărescu & Nalbant, 1995). ***Traccatichthys*** contains five typical riverine species distributed along the coastal regions of southeast China and north to middle Vietnam (Prokofiev, 2004; Du et al., 2012). ***Tuberoschistura*** contains two valid species; *T. cambodgiensis* is found in the lower Mekong basin and *T. baenzigeri* on the Malay Peninsula and in the Chao Phraya basin. *Tuberoschistura* was named for the presence of small tubercles on the barbels, cheeks, snout and top of the head (Kottelat, 1983, 1990).

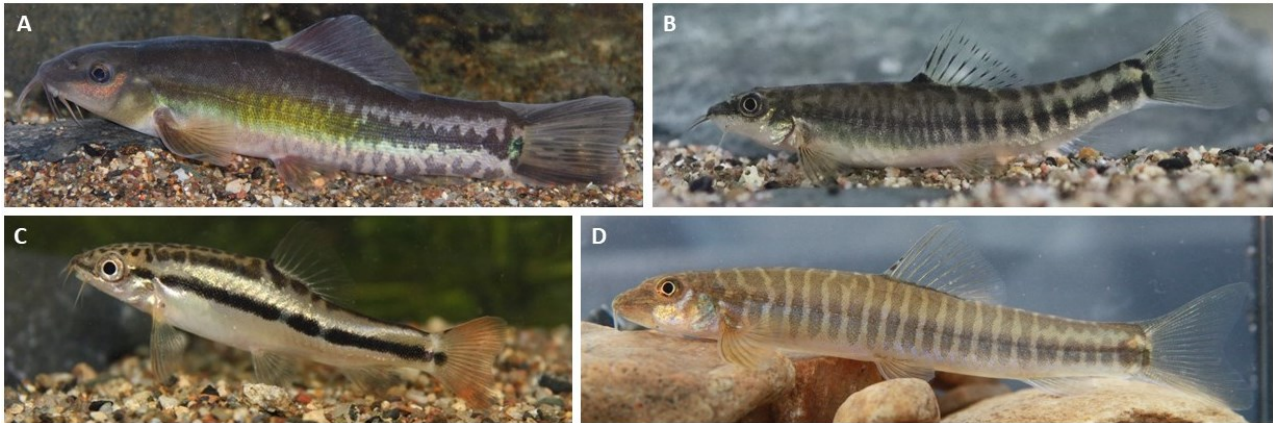


FIG.16. Pictures of selected species of ‘medium-rich’ genera. A) *Traccatichthys taeniatus*; B) *Pteronemacheilus* sp.; C) *Petruichthys brevis*; D) *Neonoemacheilus labeosus* (photos J. Bohlen/V. Šlechtová).

Nemacheilidae includes ten monotypic genera, characterized by unique morphological, ecological, or biogeographic attributes. ***Draconectes*** is represented by the troglomorphic species *D. narinus* (Kottelat, 2012b), endemic to a small cave on Van Gio Island in Vietnam. Up to now, no DNA sample was available, therefore its phylogenetic position could not be clarified. The genus ***Kapuasia***, was recently erected (Tan & Kottelat, 2024) for the species *K. maculiceps* from the Kapuas River in western Borneo. Initially described as *Schistura maculiceps* by Roberts (1986) based on a single collection from 1976, this enigmatic species exhibits morphological similarities to Indochinese taxa. However, no species with such morphology is known from the Malay Peninsula or from other Sunda Islands, making the presence of *K. maculiceps* on Borneo a mystery. In 2023, additional specimens of *K. maculiceps* were collected (Kottelat & Tan, 2024). ***Kayahschistura*** comprises a single troglomorphic species, *K. lokalayensis*, endemic to the Lokalay Loku Gu Cave in Myanmar. This species represents the first described cavefish from Myanmar (Kottelat & Grego, 2020). ***Malihkaia***, another recently described genus, with single species *M. aligera* endemic to the Mali Hka River in the northernmost Kachin State in Myanmar was discovered in 2014. The type series includes the only 5 specimens (3 males and 2 females) that were collected. The species is characterised by strongly furrowed lips and unique sexual dimorphism - pectoral fins of males are rigid and greatly elongated, with the first branched ray strongly widened and prolonged (Kottelat, 2017d). ***Nemachilichthys*** represented by *N. ruppelli* (originally *N. rupelli*) is endemic of the Indian Western Ghats. The correct spelling of the species name remains debated, with "*rupelli*" (e.g. Kottelat, 2012a; Keskar et al., 2015) and "*rueppelli*" (e.g. Keskar et al., 2016; Kullander, 2016; Bleher, 2018) both appearing in literature. The genus is diagnosed by an elongated snout, comparably huge eyes and a regularly barred colouration pattern (Keskar et al., 2015).

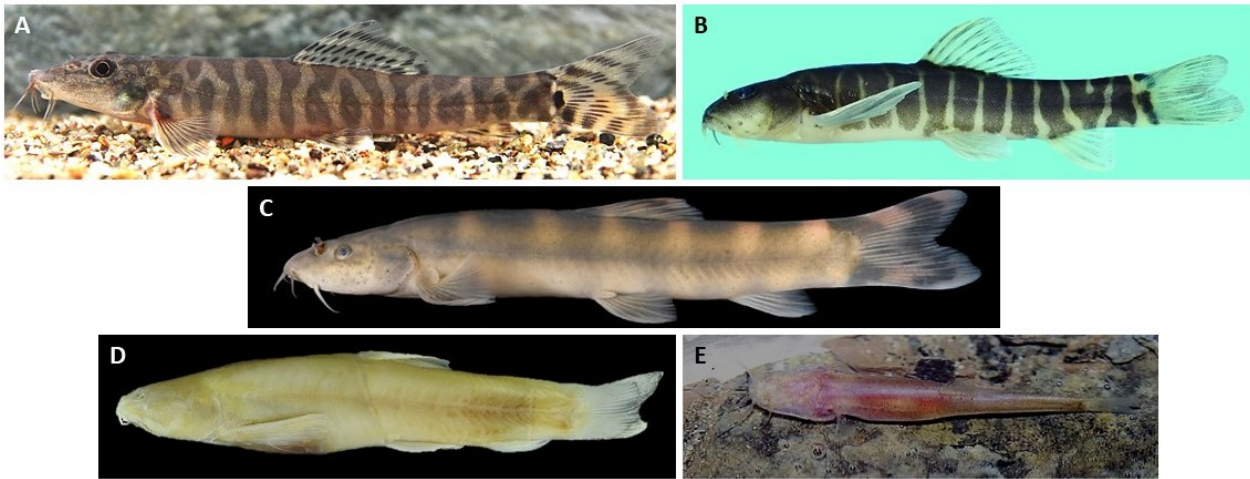


FIG.17. Pictures of selected monotypic genera. A) *Nemachilichthys rueppelli* (photo J. Bohlen); B) *Malihkaia aligera* (photo from Kottelat, 2017); C) *Kapuasia maculiceps* (photo from Kottelat & Tan, 2024); D) *Kayahschistura lokalayensis* (photo from Kottelat & Grego, 2020) ; E) *Draconectes narinosus* (photo B. Sket, from Kottelat, 2012b).

Nun represented by *N. galilaeus* is known from Jordan River drainage in Israel. While originally it was described from the Sea of Galilee, the exact type locality is unclear, with Huleh Lake as a more likely origin. *N. galilaeus* is characterised by 6 branched anal fin rays, 9-11 branched dorsal fin rays. This species is considered extinct in Israel (Bănărescu et al., 1982). The taxonomic status of a potentially conspecific population in Syria remained unresolved (Prokofiev & Golubtsov, 2013). **Protonemacheilus** is restricted to the upper Irrawaddy River drainage (Yang & Chu, 1990). The only species *P. longipectoralis* remains enigmatic due to the lack of molecular data that could enable to find its position in the nemacheilid phylogeny. Morphological similarities to *Pteronemacheilus* have been noted (Kottelat, 2013). **Sasanidus**, represented by *S. kermanshahensis*, is endemic to the Karkheh and Karun drainages in Iran. It is characterised by a marbled or mottled pigmentation pattern with an irregularly shaped midlateral stripe. Phylogenetic analyses based on COI gene, have placed *Sasanidus* in a distant relation to other Near East genera (Freyhof et al., 2016). **Speonectes** is represented by troglomorphic species *S. tiomanensis* endemic to a Tioman Island, located southeast of the Malay Peninsula (Eschmeyer, 2024). Although it shares morphological similarities with *Sundoreonectes*, genetic analyses revealed a distinct lineage, therefore new genus was established for *S. tiomanensis* (Kottelat, 2012a). **Sphaerophysa** represented by *S. dianchiensis*, endemic to Dianchi (Chi) Lake in Kunming City (Yunnan Province, China) is currently classified as critically endangered by IUCN. However, the species is rather presumed as extinct due to the severe degradation of the lake. Although Dianchi Lake was in the middle of the 20. Century home for 25 fish species (10 of them endemic), it has experienced a catastrophic decline in fish diversity (at the

turn of the century only 4 species and 1 of the 9 endemic ones left). Due to the strong pollution, in Dianchi Lake nowadays survive only a few resilient species, such as *Carassius* and *Misgurnus*, and *Sphaerophysa* is probably lost forever (Wang et al., 2013).

Aims of the study

The primary aim of the study was to provide the first comprehensive phylogenetic reconstruction of the family Nemacheilidae on the base of molecular data. Additional objectives included reconstructing the evolutionary history of selected nemacheilid groups and identifying key factors influencing their diversification. The selection of these species groups was based on available literature, distinctive morphological characters, or specific ecology. Species descriptions came as a natural outcome of the detailed phylogenetic analyses.

Research trajectory

Although the phylogenetic reconstruction of the family Nemacheilidae was the most recently completed study, it is presented first in this thesis (as a preprint), as it represents the core of the project. The primary focus throughout the research was on this topic. However, the search for additional material was a never-ending process, with important species from different genera or distinct localities continuously emerging from our cooperation partners. Consequently, the ‘final’ dataset was repeatedly expanded. Although the initial plan was to complete the phylogenetic reconstruction quickly using the material we already had, no one wanted to miss the opportunity to make the dataset more comprehensive.

Following the main phylogenetic reconstruction of the entire family Nemacheilidae, I present three publications that focus on the phylogeny, biogeography and evolutionary history of certain species-groups within Nemacheilidae. The first paper addresses *Paracanthocobitis zonalternans*, a species that inhabits the lower parts of rivers and was previously considered to be a single widespread species. However, some populations were suspected of “looking somewhat different”. The initial objectives of the study were to explore the intraspecific diversity, reconstruct its phylogeography, and assess impact of biogeographical, geological and climatic events on its population structure.

Another paper focused on the *Schistura cincticauda/robertsi* complex, inhabiting mainly the headwaters of small forest streams. Given the extremely small and isolated populations in these habitats, rapid diversification on small geographic scale due to genetic drift was hypothesised. Therefore, the main aim was to investigate how the specific lifestyle of these species is reflected in the genetic divergence within the complex and to determine species boundaries. Phylogenetic

analyses revealed ten distinct clades, five of them corresponding to previously described species, while the other five representing species new to science.

The work on these two studies began in the frame of my master's thesis. However, the analyses continued and were finalised and published during my PhD research, therefore the published papers are included in the present thesis.

The third paper investigated the proposed *Schistura poculi* species group, which was postulated to include species characterised by a unique pigmentation pattern. The primary aim was to determine the monophyly of this group and assess whether the shared pigmentation pattern represented a synapomorphy. The result did not support the monophyly of the proposed group, moreover, the analyses revealed the presence of multiple undescribed species.

As last, I present two publications addressing taxonomic questions related to the analysed species groups. One on them, stemming from the *Paracanthocobitis* study, describes one of the most genetically and morphologically distinct lineage as a new species, *Paracanthocobitis epimekes*.

The second taxonomic publication provides a revision of the *Schistura cincticauda* species group with description of five new species. Additionally, an identification key for all species in this group has been provided. This species group was initially referred to as '*Schistura robertsi*' group in the original phylogenetic paper, but the name was corrected to *Schistura cincticauda* to reflect the first described species in the complex.

General methodology

Sampling: The majority of fish samples used in this doctoral thesis was present in the fish collection of IAPG. They originate from field trips between the years 2005 and 2013, from professional fish exporters in Thailand and Myanmar, as well as from a professional field guide in Thailand. In nearly all cases the collection localities were known. A significant bunch of samples was provided by Dr. Maurice Kottelat (Cornoll, Switzerland). Several samples were obtained also from Dr. Jörg Freyhof (Berlin, Germany), Dr. Heok Hui Tan (Singapore) and about 12 further colleagues. Samples fixed in 96% ethanol were designated for genetic analyses, while formalin-fixed specimens were reserved for morphological studies and potential taxonomic implications (e.g. comparative material or type specimens for species descriptions).

Laboratory procedures: Genomic DNA was typically extracted from fin or muscle tissue either using commercial kits, such as Dneasy Blood & Tissue kit (QIAGEN), NucleoSpin Tissue kit (MACHEREY-NAGEL) or Tissue/Blood DNA Mini Kit (Geneaid). In cases the commercial kits failed to yield sufficient DNA concentration or quality, the phenol – chloroform extraction method (Sambrook et al., 1989) was employed.

All polymerase chain reaction (PCR) amplifications were performed following a well-established protocol of Laboratory of Fish Genetics (LFG). Each 25 µl reaction mixture contained PCR water, PCR Blue Buffer, MgCl₂, PCR enhancer, PCR dNTP mix, Taq DNA polymerase Unis (all chemicals by Top–Bio), and of forward and reverse primers (Generi Biotech). Thermal cycling was carried out on DNA Engine Peltier Thermal Cyclers (Bio-Rad). For sequencing, approximately half of the PCR products were sent to MACROGEN Inc. or SEQme s.r.o. for professional service. The remaining products were purified using QIAquick PCR purification kit (QIAGEN) and sequenced on an ABI Prism 3130 GA in the LFG. Sanger sequencing employed either BigDye™ Terminator Cycle Sequencing Ready Reaction Kit 1.1 (Applied Biosystems) or BrightDye® Terminator Cycle Sequencing Kit 3.1 (Carolina Biosystems). The sequencing PCR reactions typically used the same primers as the initial PCR amplification. In some cases, internal primers were also employed for sequencing.

Sequence data preparation: Sequence assembly and alignment: Chromatograms were initially checked and assembled using SeqMan II module of the DNA Star software package (LASERGENE). Single gene alignments were performed in BioEdit (Hall, 1999) using the ClustalW (Larkin et al., 2007) multiple alignment algorithms. Alignments typically included original data, in

some cases alongside sequences downloaded from GenBank. When possible, single gene alignments were concatenated in PhyloSuite v1.2.2 (Zhang et al., 2020).

Initial analyses in MEGA: Ready alignments were imported into MEGA v. 7.0 (Kumar et al., 2016) for preliminary analyses. Here, Neighbour-Joining trees were prepared to identify any potential sample mix-ups within the datasets. Additionally, MEGA was used to calculate the numbers of variable and parsimony-informative positions, providing a measure of sequence variation. For studies with deeper taxonomic focus, pairwise genetic distances were estimated and converted into percentages between the lineages (for better comparison of our findings with those reported in other studies).

Phylogenetic analyses: Model selection and tree reconstruction: Partition Finder 2 (Lanfaer et al., 2016) implemented in PhyloSuite 1.2.2 (Zhang et al., 2020) was employed to estimate the best-fit substitution models for each data partition (typically genes and codon positions). The model selection based on the corrected Akaike Information Criterion (AICc). Phylogenetic analyses were performed using both Bayesian inference (BI) and maximum likelihood (ML) approaches. For BI, MrBayes 3.2 (Ronquist & Huelsenbeck, 2003) via CIPRES Science Gateway (Miller et al., 2010) was the primary tool. Datasets were partitioned into genes and codon positions and the parameters were assigned based on the best-fit models identified earlier by PartitionFinder 2. All analyses were set to 10 – 20 million in of generations and 4, 6 or 8 parallel Metropolis Coupled Markov Chains Monte Carlo (MCMCMC). Tracer v 1.7.1 (Rambaut et al., 2018) was used to assess convergence and to ensure sufficient effective sampling size (ESS) for all parameters (>200). Trees corresponding to relative burnin of 20% were discarded and from the remaining trees 50% majority rule consensus trees were built. The partitioned ML analysis was performed using IQ-TREE (Nguyen et al., 2015) implemented in PhyloSuite. Datasets were partitioned as described earlier and the best-fit models IQ-TREE estimated automatically using ModelFinder (Kalyaanamoorthy et al., 2017) within PhyloSuite prior to the tree reconstructions Node support values were obtained by ultrafast bootstrap replicates (UFBoot) (Hoang et al., 2018).

Divergence time estimations: The ages of cladogenetic events were estimated in BEAST 2.6.0 (Bouckaert et al., 2014) via CIPRES Science Gateway. For calibration, several biogeographical or geological events as well as some internal or external fossil records were used. Similar to previously described analyses, the reliability of the results was checked in Tracer 1.7.1 to ensure sufficient ESS for all model parameters (>200). Maximum clade credibility (MCC) trees were built in TreeAnnotator 2.6.0 (Rambaut & Drummond, 2010) after discarding a portion 10 – 25 % of initial

trees, considering them part of the burnin. Final trees were visualised in FigTree 1.4.4 (Rambaut, 2019).

Species trees: For reconstructing of the species trees, primarily a multi-species coalescent approach in StarBEAST2 (Ogilvie et al., 2017) implemented in BEAST 2.6.0 (Bouckaert et al., 2014) was applied. This approach infers both gene and species trees simultaneously from multiple sequence alignment. As described previously, the results were checked in Tracer and MCC trees generated in TreeAnnotator. In some cases, also calibrated species trees were reconstructed for divergence time estimations. Occasionally, an alternative method called the Accurate Species Tree ALgorithm (ASTRAL III) (Zhang et al., 2018) was employed. For ASTRAL species trees, unrooted single-gene ML trees reconstructed in IQ-TREE (within PhyloSuite) were used as input file.

Biogeographical reconstructions: Two of the articles included within this thesis also incorporate biogeographical analysis. These analyses were conducted using the BioGeoBEARS package (Matzke, 2013) implemented in RASP 4.0 (Reconstruct Ancestral Stage in 498 Phylogenies, Yu et al., 2015). The definition of biogeographic units varied depending on the scale of the study. In case of family-level focus, the biogeographic regions were defined based on wider drainage areas separated by major mountain ridges (Šlechtová et al., 2024). When reconstructing the biogeographical history of a smaller species group, the biogeographic regions were defined according to local drainage areas and incorporated known differences in freshwater fish composition (Bohlen et al., 2020). Following the definition of regions, six biogeographic models (DEC, DEC+J, DIVALIKE, DIVALIKE+J, BAYAREALIKE, BAYAREALIKE+J) were compared and their fit was evaluated using Likelihood Ratio Test (LRT) p-values. Typically, the best-model was applied, in some cases all models were applied and the results compared.

Morphological analyses and species descriptions: External morphological characters were primarily selected based on established works by Kottelat (1990, 2000) and former descriptions of related nemacheilid fish species. Additionally, for some species we obtained micro-X-ray pictures of selected specimens from Dr. Milan Petrtyl from the Czech University of Life Sciences in Prague, and we used them for evaluation of osteological characters. Species descriptions mostly follow Kottelat (2012, 2013, 2017a-d and 2019).

For detailed information on material and methods used in each study, please refer to the corresponding papers within this thesis.

Conclusions

The results of the articles included in this thesis represent a valuable amount of new knowledge about the family Nemacheilidae. It includes the first comprehensive phylogenetic reconstruction, containing nearly 300 species across 36 genera and identifying six major clades named according to their geographical distribution. Additionally, this study provides the first detailed reconstruction of the biogeographical, geological and climatic history of Eurasia during most of Cenozoic era on the example of the freshwater fish family Nemacheilidae.

The three papers focused on species groups revealed the existence significantly higher diversity within Nemacheilidae, reflecting the fact that 200 new nemacheilid species and 13 genera were described during the last decade. The analysis of the *Paracanthocobitis* study highlighted the impact of climatic changes, particularly global sea level fluctuations, on the evolution of lowland freshwater fauna. In contrast, the *Schistura cincticauda* group demonstrated the effect of genetic drift in mainly allopatric populations. These different results in two nemacheilid species-groups within a similar geographic region underline that evolution is influenced by a variety of factors, and that evolutionary pathways are much more complex than might be apparent without detailed studies.

The last two articles together describe six new species and include comments on additional taxa that are in need of formal description.

The biogeographical reconstructions and time estimations presented across the articles reveal a significant impact of various global or local known biogeographical, geological and climatic events on the evolutionary history of the family Nemacheilidae.

Ongoing studies and future research perspectives

Taxonomic revision of the family Nemacheilidae with special focus on genera on the base of molecular phylogeny: Recent phylogenetic studies revealed that many genera within Nemacheilidae are polyphyletic or paraphyletic. However, our phylogenetic analyses indicate that the situation is even more complex than previously expected. For example, species assigned to the genus *Schistura* are dispersed across more than 15 lineages within our phylogeny, intermixing with many other genera. Some other genera, such as *Mustura*, *Nemacheilus*, *Sectoria* and *Triplophysa*, are also polyphyletic. A manuscript currently in preparation aims to map the existing genera onto our phylogenetic reconstruction and to discuss strategies to address the extensive polyphyly of genera within Nemacheilidae.

An updated phylogeny of the loach genus *Nemacheilus*: The most recent phylogenetic overview of the nemacheilid genus *Nemacheilus* was published by Šlechtová et al. (2022). Since then, many additional samples of various *Nemacheilus* species have been obtained. The analyses revealed a new major clade within the genus, an older origin of *Nemacheilus*, and several undescribed species. Updated phylogenetic analyses and related investigations have been completed, and a manuscript will be prepared.

Extensive radiation of the loach genus *Schistura* in Indochinese region: This manuscript, which has been in preparation for some time, focuses on the largest species complex within Nemacheilidae, specifically within the Indochinese clade. The dataset includes sequences of approximately 400 fishes representing the Indochinese nemacheilid diversity. The results clearly indicate that this diversity is result of a rapid radiation associated with an exceptional geographic expansion of the group. We expect that the study will reveal dozens of undescribed species in this group.

Phylogeny and biogeography of Nemacheilidae of the Northern clade: The Northern clade is one of the main clades within Nemacheilidae, containing the most widespread genus *Barbatula* and the second most diverse genus *Triplophysa*. We have collected samples representing much of the diversity within the majority of the clade's distribution area. Moreover, big amount of data is available on GenBank. This study aims to reconstruct the colonisation history of Europe and further details of the phylogeny of this clade.

Species descriptions and taxonomic revisions: Nemacheilidae indeed include a significant amount of undiscovered diversity. Our collection at LFG includes dozens of undescribed species from this family. Documenting this diversity through species descriptions is needed for cataloguing the existing diversity and highlighting the rapid decrease of biodiversity caused by factors such as habitat destruction. Species descriptions and taxonomic revisions will continue to be component of our future work.

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