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Phylogenetic position of the fish genus *Ellopostoma* (Teleostei: Cypriniformes) using molecular genetic data

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We investigated the phylogenetic position of *Ellopostoma* based on nuclear sequence data (RAG-1 gene). *Ellopostoma* is a member of the superfamily Cobitoidea (loaches) of Cypriniformes, but does not belong to any of the currently recognised families. It represents an independent lineage, recognised as a distinct new family Ellopostomatidae, characterized by a squarish and oblique snout, a minute protrusible mouth, a single pair of barbels, large eyes and 35-38 pharyngeal teeth.

Introduction

With about 3800 recognised species, the freshwater fish order Cypriniformes (Osteichthyes: Teleostei) is one of the largest recognised to date among vertebrates. It is divided into two main lineages, the superfamilies Cyprinoidea (carps, minnows and related fishes) and Cobitoidea (loaches and related fishes) (Nelson, 2006). Within Cobitoidea seven lineages are recognizable (called families by e. g., Šlechtová et al., 2007; Chen & Mayden, 2009). In the past, a number of genera were placed in Cobitoidea whose phylogenetic position were controversial. The relationships of most have been elucidated (Šlechtová et al., 2007). However, one enigmatic genus, *Ellopostoma,* was not included into the recent phylogenetic studies since no material was available. Up to now, only five publications report the finding of fishes of this genus: Vaillant (1902) described *E. megalomycter* on the basis of three specimens from the middle stretches of the Kapuas River in western Borneo. It is only in 1976 that the species was collected again, also in the Kapuas (Roberts, 1989). Kottelat (1989) recorded the presence of an unnamed *Ellopostoma* from the Malay Peninsula [Tapi River, Thailand], later described by Tan & Lim (2002) as *E. mystax*. Kottelat & Widjanarti (2005) provide additional records of *E. megalomycter*, also in the Kapuas drainage.

Because of its unique morphological features, the phylogenetic position of *Ellopostoma* has been perplexing since its very discovery. Vaillant (1902) described the first species as a member of *Aperioptus*, a genus known from a drawing of a specimen from “Borneo” (Richardson, 1848), which has not been preserved and which has never been identified with any fish species known to-date. But Vaillant was not really certain of his conclusion and while describing the species as an *Aperioptus* he proposed the genus name *Ellopostoma*, “should his *A. megalomycter* be found not to be

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Fig. 1. *Ellopostoma mystax,* 37 mm SL, not preserved; Thailand, Surat Thani province: River Tapi.

an *Aperioptus*". He placed *E. megalomycter* in the family Cobitidae, at that time almost equivalent to today's Cobitoidea. Weber & de Beaufort (1916) disagreed with the placement of *Ellopostoma* in Cobitidae [today's Cobitoidea] but were "not able to say, to which family of Cyprinidae [sic pro Cyprinoidea (today's Cypriniformes)] these probably young fish belong". Roberts (1972) re-examined the three type specimens and pointed out the close resemblance to the African Kneriidae (order Gonorhynchiformes) as well as to Nemacheilidae. However, at the end of his comparison he found *Ellopostoma* impossible to be classified. Later, after seeing fresh material, Roberts (1989) suggested that *Ellopostoma* belongs to the loaches [Cobitoidea], but stated that the relationships to other loaches remain unclear. Kottelat (1989), Kottelat et al. (1993) and Tan & Lim (2002) considered *Ellopostoma* to belong to Balitoridae (which then included genera now placed in the families Balitoridae, Vaillantellidae and Nemacheilidae). Bănărescu & Nalbant (1995) placed *Ellopostoma* in Nemacheilidae. These divergent hypotheses on the possible relationships of *Ellopostoma* result partly from the lack of material to investigate its morphology and partly from the fact that nobody has investigated the problem.

Morphologically, *Ellopostoma* differs from all other loaches by the presence of a single pair of barbels (versus three pairs in other loaches or no barbels in one species), the morphology of the snout ("unlike that in any other teleost"; Roberts, 1972), the very large eyes and nostrils and a small, ventral, protractile mouth with an extremely small gape (the name *Ellopostoma* means mouth of sturgeon) (Roberts, 1972) (Fig. 1). Although Roberts (1972) described a number of morphological characteristics of *Ellopostoma*, the material at his disposal was of poor quality and consequently he could not describe with enough details a number of characters, e.g. the presence of Weberian apparatus, the swim bladder, and the barbels. Roberts (1972) compared the characters of *Ellopostoma* with those published for Kneriidae and a few loaches, but he did not make a comparison with all lineages of Cobitoidea (those we recognise here as families) and therefore remained unable to conclude on the taxonomic position of *Ellopostoma*.

We recently collected live specimens of *E. mystax* that allow the first study of its DNA. In the present study we aim to reconstruct the phylogenetic relationships of *Ellopostoma* on the base of the nuclear RAG-1 gene.

Material and methods

The molecular analyses includes nine specimens of *E. mystax* from Thailand, Surat Thani province, River Tapi (GeneBank accession numbers EU562197-EU562205), 63 specimens of Cobitoidea representing all recently recognised lineages (Balitoridae, Botiidae, Catostomidae, Cobitidae,

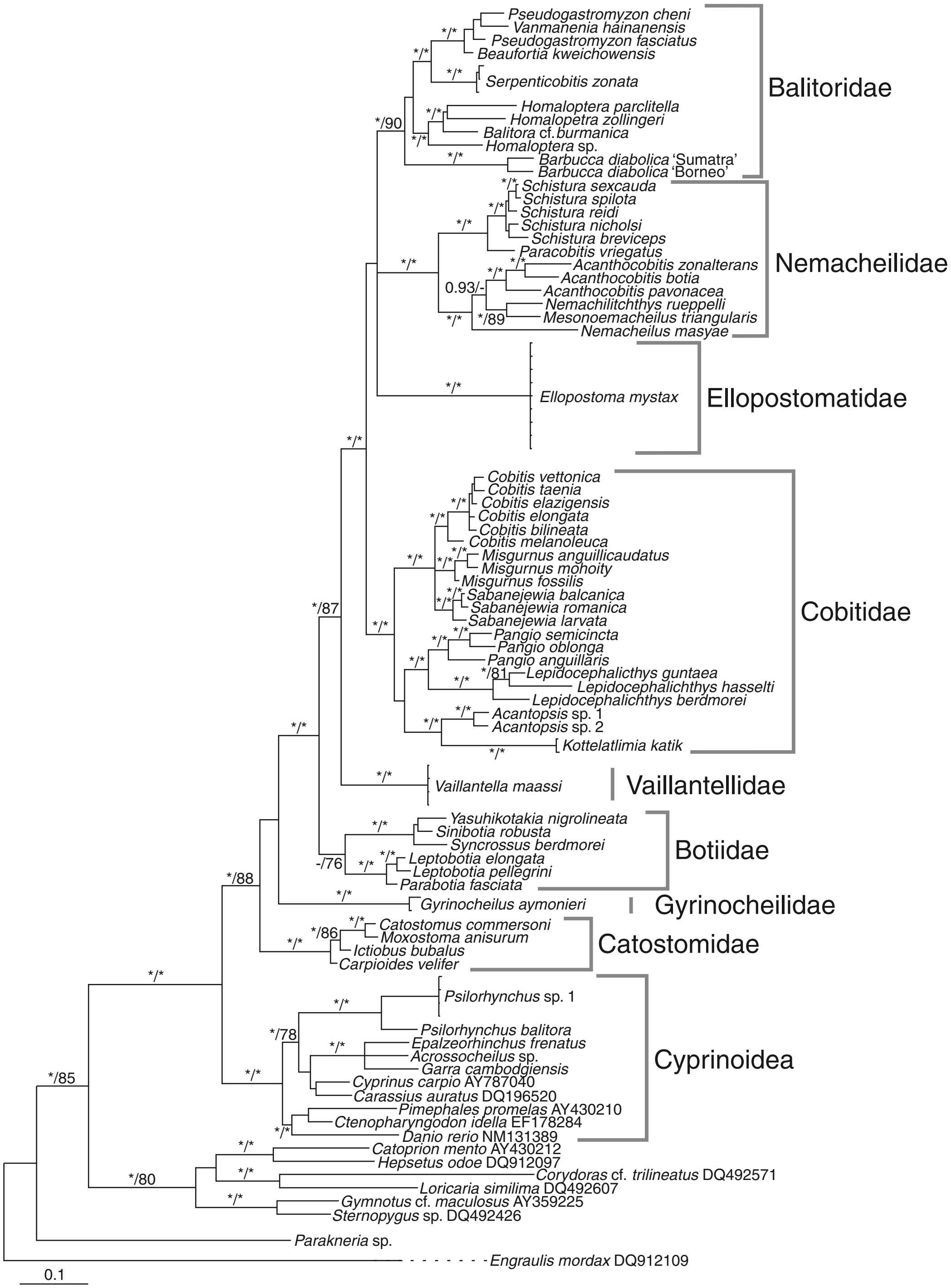

Fig. 2. Molecular phylogeny of the Cobitoidea. Majority rule consensus tree resulting from the Bayesian analysis of the RAG-1 gene dataset. At the nodes are Bayesian posterior probabilities before the slash and Maximum Likelihood bootstrap values behind the slash. Asterisks indicate posterior probability values >0.95 and bootstrap values >90.

Gyrinocheilidae, Nemacheilidae, Vaillantellidae) as well as 13 specimens of Cyprinidae. The geographic origin of these fishes and their Genebank accession numbers are given in Šlechtová et al. (2007). As Roberts (1972) suggested similarities between *Ellopostoma* and Kneriidae, a specimen of *Parakneria marmorata* was included (accession number EU562196). To give a phylogenetic frame to this widened sample set, we included the remaining orders of Ostariophysi as well as *Engraulis* (Clupeomorpha: Clupeiformes) as outgroup.

An approximately 900 bp long stretch of the nuclear gene RAG-1 was sequenced from ethanol preserved tissue following Šlechtová et al. (2007). This gene has been demonstrated repeatedly to serve as well-suited marker to resolve phylogenetic relationships at the taxonomic level in focus here and to bring reliable results in single-gene studies (López et al., 2004; Šlechtová et al., 2007; Sullivan et al., 2006). The obtained sequences were assembled and checked by eye using SeqMan II 5.05 module of the Lesergene software package (DNAStar). The multiple sequence alignment was conducted in BioEdit 7.0.5.3 using Clustal W algorithm.

Nucleotide composition and χ^2 test of homogeneity of base frequencies were computed in PAUP* v. 4.b10 (Swofford, 2002) for all positions as well as for each codon position separately. All three codon positions were examined for saturation level by plotting the absolute number of sequence differences (transitions and transversions separately) against the uncorrected *p*-distances. The slopes of the regression lines were used to evaluate the levels of saturation.

To assess the systematic position of *Ellopostoma*, we conducted a Bayesian analyses (BI) with MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) and a maximum likelihood analysis (ML) with GARLI v. 0.95 (Zwickl, 2006). Modeltest 3.06 (Posada & Crandall, 1998) was used in order to determine the best-fit model of nucleotide evolution for the given dataset. For BI, six Markov Chains Monte Carlo were run simultaneously for 2×10^6 generations starting with randomly generated trees under the GTR+I+Γ model as estimated by Modeltest. The dataset was partitioned according to codon position and the model parameters were estimated for each data partition separately. The trees and likelihood scores were sampled each 100 generations. The burn-in period was estimated by plotting the log-likelihood scores of all 20,000 sampled trees against generation times. The values reached stability after ca. 50,000 generations; the corresponding trees were discarded from further analyses. The remaining (post burn-in) trees were used to build a 50 % majority consensus tree. The posterior probabilities reflect the frequency of each particular clade in the sampled trees.

For the ML analysis, parameters were set to GTR+I+Γ model with 6 rate categories, rate heterogeneity across sites, proportion of invariable sites and four different base frequencies. The parameter values were estimated by GARLI. We used 1000 nonparametric bootstrap resamplings to assess the statistical support of the nodes. From the resulting trees we built a 50 % majority rule consensus tree in PAUP* to obtain the bootstrap values.

Results

The final alignment contained 903 bp of 93 specimens. In the whole dataset there is a total of 472 (52 %) variable sites, of which 416 (46 %) are parsimony informative. Within the ingroup (cypriniform taxa), 425 (47 %) of variable and 382 (42 %) of parsimony informative nucleotide positions are detected. No variability is observed within the nine sequences of *Ellopostoma*. The estimated Ti/Tv ratio is 1.8. An inspection of the saturation plots reveals that no trend towards saturation is apparent in any of the codon positions (data not shown). No compositional bias is observed across all taxa for all three codon positions ($\chi^2 = 278.3$, d.f. = 279, $P > 0.5$), when analysed separately, significant heterogeneity in the nucleotide composition is observed only at the third codon position ($\chi^2 = 647.6$, d.f. = 279, $P < 0.001$). Mean base compositions are 23.8 % A, 24.4 % C, 27.7 % G, 24.1 % T.

In the presented data set, our analyses identifies Cypriniformes as monophyletic lineage, forming the sister lineage to a lineage comprised by the orders Siluriformes, Gymnotiformes and Characiformes (Fig. 2). The order Gonorhynchiformes represents the sister lineage to all other Ostariophysi. Cypriniformes split into two subgroups corresponding to Cobitoidea and Cyprinoidea. Cobitoidea comprises eight major monophyletic lineages, seven of them representing the formerly identified families (Šlechtová et al., 2007), the eighth being formed by *Ellopostoma*. Within Cobitoidea, Catostomidae and Gyrinocheilidae represent the most basal clades. Loaches sensu stricto comprise of Botiidae and Vaillantellidae and four lineages with unresolved phylogeny: Cobitidae, Balitoridae, Nemacheilidae and *Ellopostoma*.

Discussion

The results of our analyses show that the genus *Ellopostoma* belongs to the superfamily Cobitoidea and is most closely related to the loach families Nemacheilidae, Balitoridae and Cobitidae. However, it cannot be assigned to any of these families, but represents a distinct lineage. Although *Ellopostoma* seems more closely related to Nemacheilidae and Balitoridae than to Cobitidae, this relation was not statistically supported, thus at present we have to consider the four lineages to form a polytomy. The distinctness of *Ellopostoma* from the other loaches explains why former studies had difficulties to assign *Ellopostoma* to any existing family and why there had been little agreement in former opinions to which family *Ellopostoma* might belong.

On the base of our investigations we consider *Ellopostoma* as a distinct lineage among Cobitoidea that does not belong to any of the families recognised up to now. Consequently, we place it in a family of its own, Ellopostomatidae fam. nov. (type genus *Ellopostoma* Vaillant, 1902). The morphological characters that are diagnostic for the new family are: 1) snout squarish and oblique (versus rounded or pointed with ventral margin horizontal in all other Cobitoidea; 2) mouth very small (width of mouth 7-8 times in head width, versus 1.5-4 times) and highly protrusible (versus not protrusible); 3) very large eyes, 26-36 % of lateral head length (versus 8-27 or no eyes); 4) a single pair of barbels (versus 3-5 pairs of barbels or no barbels); and 5) 35-38 phrayngeal teeth (versus 8-25). A more detailed morphological analysis is in preparation (see also Bohlen et al., 2007).

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