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Molecular phylogeny of the Southeast Asian freshwater fish family Botiidae (Teleostei: Cobitoidea) and the origin of polyploidy in their evolution

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Abstract

The freshwater fish family Botiidae is represented by seven genera on the Indian subcontinent and in East and Southeast Asia and includes diploid as well as evolutionary tetraploid species. We present a phylogeny of Botiidae including 33 species representing all described genera using the mitochondrial cytochrome *b* and 12s rRNA genes to reconstruct the phylogenetic relationships among the genera and to estimate the number of polyploidisation events during their evolution. Our results show two major lineages, the subfamilies Leptobotiinae with the genera *Leptobotia* and *Parabotia* and Botiinae with the genera *Botia*, *Chromobotia*, *Sinibotia*, *Syncrossus*, and *Yasuhikotakia*. Our results suggest that two species that were traditionally placed into the genus *Yasuhikotakia* form a monophyletic lineage with the species of *Sinibotia*. A review of the data on the ploidy level of the included species shows all diploid species to belong to Leptobotiinae and all tetraploid species to Botiinae. A single polyploidisation event can therefore be hypothesised to have occurred in the ancestral lineage leading to the Botiinae.

Keywords: Loach fishes; Tetraploidy; Polyploidisation; Cytochrome *b*; 12s rRNA

1. Introduction

Botiid loaches represent an interesting model to study the role of polyploidisation in vertebrate evolution since they include diploid (with $2n = 50$ chromosomes) as well as evolutionary tetraploid species ($2n = 98$–100 chromosomes). From the 26 species surveyed by Suzuki and Taki (1996) in a review of ploidy level in botiid fishes, 11 were diploid and 15 tetraploid. Polyploidisation is well known as an important evolutionary force in plants and indications for its importance in the evolution of animals are constantly accumulating (Le Comber and Smith, 2004; Soltis and Soltis, 1995, 1999). Polyploidisation events seem to be more common than they had been until recently believed (Leggatt and Iwama, 2003; Soltis and Soltis, 1999) and recurrent formations of polyploid taxa were already called the norm rather than the exception (Soltis and Soltis, 1999). Examples of fish groups, in which changes in ploidy level have been already identified as key events in their evolution include Acipenseridae (Ludwig et al., 2001), Cyprinidae (Alves et al., 2001; David et al., 2003), Catostomidae (Ueno et al., 1988), and Salmonidae (Crespi and Fulton, 2004; Phillips and Ráb, 2001). Multiple origins of polyploidy were demonstrated in African barbs (Tsigenopoulous et al., 2002) and Cobitidae (Janko et al., 2003). However, to evaluate the origin of polyploidisation within Botiidae and its contribution to its evolution, it is necessary to understand the phylogenetic relationships between the diploid and tetraploid species.

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The relationships within Botiidae and the natural lineages inside this family have been formerly discussed mainly on the basis of morphological data. For a long time, they were considered a subfamily of the family Cobitidae and believed to include only two genera, *Botia* and *Leptobotia*, the former one with three subgenera *Botia*, *Sinibotia* and *Hymenophysa* (today called *Syncrossus*) (Fang, 1936; Nalbant, 1963; Sawada, 1982; Taki, 1972). Nalbant (2002) and Kottelat (2004) used family rank for botiid loaches, genus rank for the former subgenera and described the genera *Yasuhikotakia* and *Chromobotia*. Nalbant's (2002) suggestion to include the genus *Vaillantella* as subfamily *Vaillantellinae* into the Botiidae was in disagreement with the opinions of Kottelat (1994), Roberts (1989), and Sawada (1982) that *Vaillantella* is a nemacheilid loach and was strongly refuted by Kottelat (2004). Nalbant (2002) divided his subfamily Botiinae (Botiidae without *Vaillantella*) into two tribes, Leptobotiini (containing the genera *Leptobotia*, *Parabotia*, and *Sinibotia*) and Botiini (with the genera *Botia*, *Hymenophysa*, and *Yasuhikotakia*). Recently, Tang et al. (2005) demonstrated with mtDNA data that Chinese Botiidae form a monophyletic group distinct from other loach fishes and that the genus *Sinibotia* represents the sister lineage to a lineage formed by the sister genera *Leptobotia* and *Parabotia*, but no other genera were included into their study. Since the taxonomic studies of Nalbant (2002) and Kottelat (2004) have considered *Sinibotia* to be much closer related to *Leptobotia* and *Parabotia* that other genera of Botiidae, the diversity of the family was by far not covered by the study of Tang et al. (2005). Especially the tetraploid species were strongly underrepresented in the study of Tang et al. (2005), since only three species (all *Sinibotia*) were included. To understand the phylogenetic relationships within the Botiidae and the relationship of diploid and tetraploid species, a comparison of all described genera and most species is needed.

The aim of the present study was to reconstruct the phylogenetic relationships among the genera and main lineages of the family Botiidae on the base of a wide spectrum of representatives of all described genera using sequences of mitochondrial DNA. The results of our DNA analyses are compared with the outline of the recently recognised genera that were based on morphological data. Agreement between genetic and morphological data indicates reliable groupings that most likely reflect true phylogenetic relationships. Another goal was to evaluate the phylogenetic relationships of diploid and tetraploid species in order to estimate the origin of polyploidy in the evolution of Botiidae.

2. Materials and methods

2.1. Taxon sampling

According to the most recent review (Kottelat, 2004), Botiidae include seven genera with 47 species. The present study comprises 96 individuals of 33 species, covering the diversity of the family (3 out of 5 species of *Sinibotia*, 6 out of 8 of *Botia*, 4 out of 7 of *Parabotia*, 7 out of 13 of *Leptobotia*, 7 out of 9 of *Yasuhikotakia*, all species of *Syncrossus* (5) and the monotypic *Chromobotia*) (Table 1). Due to the extensive sampling, our study covers all valid genera of Botiidae and all generic type species. Initial analyses showed that *Vaillantella* is not closely related to botiids; therefore *Vaillantella* was not included into this study. Regarding Botiidae, 24 sequences of cytochrome *b* were obtained from GenBank, two sequences were provided by K.-E. Witte; the remaining 70 sequences of cytochrome *b* and all 48 sequences of 12S rRNA are original data. As outgroup taxa of the family Cobitidae we included cytochrome *b* sequences of *Cobitis bilineata* and *Sabanejewia larvata* and 12S rRNA sequence of *Cobitis sinensis* from GenBank and two original sequences of *Sabanejewia balcanica*. Specimens of Botiidae were obtained from commercial imports. Identification of the examined fishes followed Kottelat (2001, 2004), Rainboth (1996), Roberts (1989), Taki (1972), and Yang and Chen (1992). M. Kottelat kindly checked our identification of the specimens of the genera *Syncrossus* and of *Y. sidthimunki* and *Y. nigrolineata*. Our specimens of *B. rostrata* and *B. almorhae* were identified by comparison with the syntypes of *B. rostrata* (BMNH 1855.12.26.694 and 1860.3.19.114) and *B. almorhae* (BMNH 2002.9.18.1). Voucher specimens are available in the collection of IAPG.

2.2. DNA isolation, PCR, and sequencing

Genomic DNA was isolated from fin or muscle tissue following the standard phenol–chloroform method (Sambrook et al., 1989) or with DNeasy Tissue Kit (QIAGEN).

PCR amplification was performed in 50 μl reaction volumes containing 10 mM Tris–HCl, 50 mM $(HN_4)_2SO_4$, 0.1% Triton X-100, 1.2–1.8 mM $MgCl_2$, 2 mM TMA oxalate (PCR enhancer), 10 nmol of each nucleotide, 2.5 U of *Taq* polymerase (all chemicals by Top–Bio) and 25 pmol of each primer. The PCR profile (carried out on MJ Research thermocycler) started with 2 min period of initial denaturation at 95 °C followed by 35 cycles each consisting of a denaturation step at 94 °C for 30 a primer annealing step from 52 to 57 °C for 30 s and elongation at 72 °C for 45 s. The PCR was completed by a final elongation step of 5 min at 72 °C. Problematic samples were PCR amplified in two overlapping fragments using internal primers under the same PCR conditions. PCR products were purified by ethanol precipitation or using Microcon PCR Filter Units (Millipore) and subdued to cycle sequencing employing BigDye Terminator Cycle Sequencing Kit v.3.1 (PE Applied Biosystems) according to manufacturer's instructions. Sequencing products cleaned by ethanol precipitation or with DyeEx 2.0 Spin Kit (QIAGEN) were resolved on ABI Prism 310 Genetic Analyser (Perkin Elmer). Each sample was sequenced with the same primers as used for double strand PCR amplification. Primers used in this study are listed in Table 2.