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Phylogenetic relationships of Middle American cichlids (Cichlidae, Heroini) based on combined evidence from nuclear genes, mtDNA, and morphology

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ABSTRACT

Heroine cichlids are the second largest and very diverse tribe of Neotropical cichlids, and the only cichlid group that inhabits Mesoamerica. The taxonomy of heroines is complex because monophyly of most genera has never been demonstrated, and many species groups are without applicable generic names after their removal from the catch-all genus *Cichlasoma* (sensu Regan, 1905). Hence, a robust phylogeny for the group is largely wanting. A rather complete heroine phylogeny based on *cytb* sequence data is available [Concheiro Pérez, G.A., Říčan O., Ortí G., Bermingham, E., Doadrio, I., Zardoya, R. 2007. Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae) based on sequences of the cytochrome *b* gene. Mol. Phylogenet. Evol. 43, 91–110], and in the present study, we have added and analyzed independent data sets (nuclear and morphological) to further confirm and strengthen the *cytb*-phylogenetic hypothesis. We have analyzed a combined *cytb*-nuclear (RAG1 and two S7 introns) data set of 48 species representing main heroine lineages to achieve further resolution of heroine higher taxonomic levels and a combined *cytb*-morphological data set of 92 species to stabilize generic taxonomy. The recovered phylogenies supported the circumamazonian—CAM—Heroini (sensu Concheiro Peréz et al., 2007) as a monophyletic group, that could be divided into six main clades: (1) australoheroines (the southernmost heroine genus *Australoheros*), (2) nandopsines (the Antillean genus *Nandopsis*), (3) caquetaines (including the north western Amazonian genera *Caquetaia* and *Heroina*), (4) astatheroines (including *Astatheros*, *Herotilapia* and *Rocio*), (5) amphilophines (including *Amphilophus* and related genera), and (6) herichthyines (including *Herichthyis* and related genera). Nuclear and mitochondrial data partitions arrived at highly congruent topologies. Suprageneric relationships were influenced mainly by the nuclear signal, as well as the most basal phylogenetic position of *Australoheros* within CAM heroines. The new phylogeny of the tribe Heroini provides robust framework to stabilize the taxonomy of the group and for future comparative studies on these morphologically and ecologically diverse freshwater fishes. Morphology was mostly informative at the genus level and aid in determining the monophyly and composition of heroine genera. Upon acceptance of all putative genera, as recovered in this study, the Heroini would be with 35 genera the most genus-rich clade of Neotropical cichlids.

1. Introduction

Heroine cichlids are secondary freshwater fishes that constitute an important component of the Neotropical fish fauna, especially in Mesoamerica, where they make up some 25% of the freshwater fish diversity (Bussing, 1985). They are one of the few groups of freshwater fishes that are distributed from southern South America to North America (where they cross the trans-Mexican volcanic Belt), and also are the only cichlids in the Greater Antilles. Heroine cichlids show a wide diversity of morphologies, as well as ecological and behavioral adaptations (e.g. Bussing, 1985; Martin and Bermingham, 1998; Miller et al., 2005). Moreover, they constitute a model system to study biogeography of the Neotropical region (Concheiro Pérez et al., 2007), and for instance, the Midas cichlid complex (*Amphilophus* sp.) living in crater lakes in Nicaragua has been proposed to be a model system to study sympatric speciation (Barluenga et al., 2006).

The taxonomical and nomenclatural history of the tribe Heroini is inextricably connected with that of the genus *Cichlasoma* Swainson, 1839. After the revision of Regan (1905) most heroine species were assigned to the genus *Cichlasoma*. However, Kullander (1983) recognized that *Cichlasoma* was an unnatural catch-all group, and restricted it to 12 morphologically very similar species closely

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related to the genus *Aequidens* Eigenmann & Bray, 1894 (but a sizeable portion of Mesoamerican heroines was still left in *Cichlasoma* in Kullander, 2003). At present, the genus *Cichlasoma*—somewhat ironically—is the type of Cichlasomatini. Further work (summarized in Kullander, 1998) elucidated the generic taxonomy of the Cichlasomatini, but Heroini were almost completely left out, and this chaotic situation has changed little since then. At present, the difficulty with assigning generic names to many heroines hinders evolutionary studies with these taxa, and necessarily requires resolving with confidence phylogenetic relationships among species and genera.

The monophyly of the tribe Heroini as well as its sister group relationship with the tribe Cichlasomatini, forming together the subfamily Cichlasomatinae are well supported based on both morphological and molecular grounds (Kullander, 1998; Farias et al., 1999, 2000, 2001). However, phylogenetic relationships and generic allocation of most species within the tribe Heroini are highly contentious, and far from being understood (Miller, 1966, 1996; Miller et al., 2005; Kullander, 1998, 2003; Concheiro Pérez et al., 2007).

In spite of many morphological studies published on heroine cichlids (Appendix 1), none to date has analyzed morphological characters in combination with molecular data. On the other hand, early studies utilizing molecular data were based mostly on mitochondrial (mt) cytochrome *b* (*cytb*) gene sequences, and were characterized by relatively sparse taxon samplings (Roe et al., 1997; Martin and Bermingham, 1998; Hulsey et al., 2004; Říčan and Kullander, 2006; Chakrabarty, 2006). The most inclusive molecular study (Concheiro Pérez et al., 2007) to date on heroine phylogenetic relationships included virtually all heroine lineages, but was based solely on *cytb*. Concheiro Pérez et al. (2007) showed that heroines could be divided into a paraphyletic stem lineage of Amazonian genera (referred to as the Amazonian Heroini; AM) and a monophyletic lineage (termed Circumamazonian Heroini; CAM) including all Middle American, Antillean and trans-Andean heroine cichlids as well as three *cis*-Andean South American genera, namely *Caquetaia*, *Heroina* and *Australoheros*. Moreover, the majority of Mesoamerican heroines could be placed into either one of two large suprageneric clades, the amphilophines and the herichthyines (Concheiro Pérez et al., 2007). This study set a sound starting point towards resolution of heroine phylogeny and stabilization of generic taxonomy. However, the *cytb*-based phylogeny of heroines (Concheiro Pérez et al., 2007) is characterized by rather short internodes, which require further confirmation both by analyzing more mt sequence data to find additional synapomorphies, as well as by examining independent data sets. In this regard, it is well known that phylogenetic analyses of combined mt and nuclear sequence data provide resolution not achieved by each type of data separately (e.g. Brower et al., 1996; Wiley et al., 1998; Rüber et al., 2004; Farias et al., 2000; López-Fernández et al., 2005a). Thus far, however, only two studies (Chakrabarty, 2006; Higham et al., 2007) have used nuclear sequence data to address phylogenetic relationships of Heroini, although with reduced taxon samplings. Phylogenetic studies based on morphology could be very informative together with molecular sequence data in further supporting monophyly of the different genera.

Given the urgent need of having a robust phylogeny of Mesoamerican cichlids as framework for the wealth of comparative studies ongoing on these taxa, the main goal of the present study was to strengthen and test the previous hypothesis on the phylogenetic relationships of CAM heroine cichlids based on *cytb* data (Concheiro Pérez et al., 2007) by analyzing two additional independent (nuclear and morphological) data sets using a thorough generic sampling. Two nuclear markers were studied to increase phylogenetic resolution both at deeper levels (*RAG1* gene), as well as at lower taxonomic levels (two introns of the S7 gene). In addition, phylogenetic analyses of a rather complete morphological data set were performed with the particular goal of stabilizing heroine genera.

2. Materials and methods

2.1. Taxon sampling

In order to further resolve the phylogeny of heroines, a nuclear (RAG1 and S7 introns) sequence data set, which included 48 species representing all major lineages of CAM heroines as well as most genera was compiled, and analyzed in combination with a mt *cytb* gene sequences of the same 48 species, most taken from Říčan and Kullander (2006) and Concheiro Pérez et al. (2007), and four newly sequenced species ('*Heros*' *beani*, *Theraps bocourti, Theraps irregularis* and *Theraps nourissati*).

In order to test monophyly of the different genera and stabilize heroine taxonomy at this level, a morphological data set was gathered based on an extensive review of literature coupled with a thorough study of museum specimens (Appendix 2). A total of 97 CAM heroine species representing all putative and established CAM heroine genera, as well as all type species of established CAM heroine genera were included in the phylogenetic analyses based on morphology combined with *cytb* gene sequence data (Říčan and Kullander, 2006; Concheiro Pérez et al., 2007; this paper).

In all phylogenetic analyses, geophagines, cichlasomatines and Amazonian heroines were used as outgroup taxa.

2.2. Generic placement of heroine species in this study

The present nomenclatural treatment of the more than 100 Mesoamerican heroine cichlid species most of which were formerly referred to as *Cichlasoma* (Regan, 1905) is both chaotic and frustrating (Kullander, 1983, 2003; Concheiro Pérez et al., 2007). To deal with the complex nomenclature of CAM heroines, we used the following approach. Monophyletic lineages including type species of established genera were considered valid, and their species composition was adjusted to keep the genera monophyletic. Established genera, which we found as non-monophyletic were restricted to include only the type species and the monophyletic lineage to which it belongs. The remaining lineages excluded from these previously non-monophyletic genera, and those monophyletic lineages without applicable generic names were propose to be named as new genera (we refer to these putative new genera as '*Heros*' species groups) if their monophyly was found to be a significantly better hypothesis than competing hypotheses found in the literature.

2.3. Molecular methods

DNA was extracted from small pieces of muscle or gill (10–25 mg) using the DNeasy™ Tissue Kit (QIAGEN). The complete *cytb* gene was PCR amplified in four species as previously described (Concheiro Pérez et al., 2007). The 3′ half of the RAG1 gene (1.5 kb) was PCR amplified with primers RAG1F1 5′-CTG AGC TGC AGT CAG TAC CAT AAG ATG T-3′ and RAG1R1 5′-CTG AGT CCT TGT GAG CTT CCA TRA AYT T-3′ (López et al., 2004). Two introns of the S7 ribosomal protein-coding gene were PCR amplified using primers S7RPEX1F 5′-TGGCCTCTTCCTTGGCCGTC-3′ and S7RPEX2R 5′-AACTCGTCTGGCTTTTCGCC-3′ for S7 gene intron 1 and primers S7RPEX2F 5′-AGCGCCAAAATAGTGAAGCC-3′ and S7RPEX3R 5′-GCCTTCAGGTCAGAGTTCAT-3′ for S7 gene intron 2 (Chow and Hazama, 1998).

PCR amplification of RAG1 gene was carried out with an initial denaturing step at 95 °C for 1 min, followed by 35–40 cycles of amplification (denaturing at 95 °C for 45 s, annealing at 54 °C for