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Molecular phylogeny and evolution of internal fertilization in South American seasonal cynopoeciline killifishes ☆

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ABSTRACT

Internal fertilization is a widespread mode of reproduction in chondrichthyans and tetrapods, but uncommon in actinopterygian fishes. In killifishes of the suborder Aplocheiloidei, internal fertilization is restricted to two genera, *Campellolebias* and *Cynopoecilus*, both containing species adapted to life in seasonal pools of subtropical South America and exhibiting elaborated inseminating structures. Phylogenetic studies involving these genera are scarce and limited to morphological characters and fragments of mitochondrial DNA sequences available for a few taxa, providing incongruent results and thus impeding hypotheses on the evolution of insemination and related morphological traits. We analyzed three nuclear loci (*GLYT1*, *ENC1*, *Rho*) for 13 aplocheiloid taxa obtaining the first well-supported phylogeny for cynopoecilines, thus providing a significant background to interpret evolutionary changes within the group. Like in killifishes of the suborder Cyprinodontoidei, the evolution of internal fertilization in aplocheiloids is associated with deep changes in the structure of male anal fin. The phylogenetic analyses indicate that internal fertilization corresponds to a single evolutionary event during the evolution of aplocheiloid killifishes. The analyses also indicate that male specialized muscle characters, comprising a muscular ejaculatory pump in the urogenital region and hypertrophied inclinatores and depressores anales, arose in the ancestor of the clade comprising *Campellolebias* and *Cynopoecilus*. On the other hand, anal fin specialized structures including the male inseminating tube of *Campellolebias* and the male inseminating fan of *Cynopoecilus* evolved independently in each genus.

1. Introduction

Internal fertilization is a widespread mode of reproduction in chondrichthyans and tetrapods, but uncommon in actinopterygian fishes (*e.g.*, Kardong, 2012). Among actinopterygians, the teleost fish order Cyprinodontiformes is well known by the broad occurrence of internal insemination and viviparity among three families of the suborder Cyprinodontoidei, the Anablepidae, Goodeidae, and Poeciliidae, which include popular aquarium and model experimental fish species (*e.g.*, Parenti, 1981; Meyer and Lydeard, 1993; Pollux et al., 2014). However, data on inseminating killifishes of the suborder Aplocheiloidei have been rare in the scientific literature (Costa, 1995a,b).

The Aplocheiloidei comprises a diversified group of killifishes, with over 620 species in about 45 genera and three families, occurring in tropical and subtropical areas of southern Asia, Africa and Americas (Costa, 2008a). However, internal fertilization is restricted to ten species belonging to two genera of the rivulid tribe Cynopoecilini, *Campellolebias* Vaz-Ferreira and Sierra, 1974 and *Cynopoecilus* Regan, 1912, both from subtropical South America in southern Brazil (Costa, 2002a, 2006). The Cynopoecilini is part of the subfamily Cynolebiasinae, a diversified clade of South American killifishes (Costa, 1990, 1998a), in which all included members exhibit elaborated reproductive courtship behavior patterns (Belote and Costa, 2002, 2003, 2004). In the Cynolebiasinae, including the cynopoeciline genera *Leptolebias* Myers, 1952 and *Notholebias* Costa, 2008, the most common reproductive strategy is external fertilization, consisting of the couple simultaneously spawning and fertilizing eggs in the bottom substrate after elaborated courtship behavior (Costa, 1988, 1990; Belote and Costa, 2002, 2003, 2004). However, in species of *Campellolebias* and *Cynopoecilus*, internal fertilization occurs with the couple swimming slowly at midwater, side to side, while male involves females with the dorsal and anal fins (Costa et al., 1989; Costa, 1998a). In these genera, male transfer sperm directly to female urogenital tract through specialized structures present on the male anal fin, whereas spawning occurs later with female alone

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depositing their eggs in the substratum (Costa et al., 1989; Costa, 1998a). In addition, all the Cynolebiasinae inhabit temporary pools formed in the rainy seasons (*e.g.*, Costa, 1998a), surviving during dry periods through resistant eggs in embryonic diapause buried in the substratum (*e.g.*, Wourms, 1972). Species with this specialized life style occur both in the killifish families Rivulidae, in South America, and Nothobranchiidae, in Africa, which have been often known as annual fishes (*e.g.*, Wourms, 1972; Parenti, 1981; Costa, 1998a, 2015) or more correctly as seasonal killifishes (Costa, 2002b).

Past studies on *Campellolebias* and *Cynopoecilus* were directed to taxonomy of Neotropical aplocheiloids, but also focused some aspects of morphology and behavior (Vaz-Ferreira and Sierra, 1974; Parenti, 1981; Costa et al., 1989; Costa, 1990, 1998a, 2008b). *Campellolebias* and *Cynopoecilus* have been hypothesized to be sister groups on the basis of morphological characters (Costa, 1990, 1998a). However, although specialized structures for insemination are present on the anterior portion of the male anal fin in species of both genera, structures in *Campellolebias* highly differs from those in *Cynopoecilus* (Costa, 1995a,b), making difficult to recognize homologous traits (Costa, 1998a). Cynopoecilini monophyly was corroborated in two molecular phylogenetic studies focusing in more inclusive groups of Neotropical aplocheiloids (Hrbek and Larson, 1999; Murphy et al., 1999) and using mitochondrial DNA (mt-DNA), but few cynopoeciline species were then sampled (*i.e.* three and four, respectively). *Cynopoecilus* was not sampled in Murphy et al. (1999), whereas sister group relationships between *Campellolebias* and *Cynopoecilus* indicated in morphology-based analyses (Costa, 1990, 1998a) was not supported in the mt-DNA phylogeny by Hrbek and Larson (1999). A more recent study that examined the phylogenetic position of a new species of *Cynopoecilus* (Ferrer et al., 2014) used morphological data available in the literature and not surprisingly corroborated *Campellolebias* and *Cynopoecilus* as sister groups. The objective of the present study is to provide the first multigene phylogeny for the Cynopoecilini, assessing the origin and evolution of morphological structures associated with internal insemination.

2. Materials and methods

2.1. Taxon sampling

Field studies were made between 2013 and 2014 to all known collecting sites of the Cynopoecilini in order of to obtain specimens to extract DNA. However, due to habitat decline many populations reported in previous studies (Costa, 2002a, 2006), disappeared. As a result, we obtained tissues of nine species of the Cynopoecilini representing all the main lineages except *Mucurilebias leitaoi* (Cruz and Peixoto, 1991), a basal cynopoeciline taxon endemic from northeastern Brazil that is not found since 1988 and is possibly extinct (Costa, 2014). Euthanasia followed the guidelines of the Journal of the American Veterinary Medical Association (AVMA Guidelines) (Leary et al., 2013), and European Commission DGXI (Close et al., 1996, 1997). Specimens were euthanized just after collection in a buffered solution of ethyl-3-amino-benzoat-methansulfonat (MS-222) at a concentration of 250 mg/l, for a period of 10 min or more, until completely ceasing opercular movements. Molecular data were obtained from specimens fixed just after euthanasia in 98% ethanol and later preserved in the same fixative. A list of specimens and respective GenBank accession numbers is provided in Table S1. Morphological comparisons were mostly based on specimens already available in fish collections as listed in former studies (Costa, 2002a, 2006), deposited in the ichthyological collection of the Institute of Biology, Federal University of Rio de Janeiro, Rio de Janeiro (UFRJ).

2.2. DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from muscle tissue of the right side of the caudal peduncle using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer instructions. To amplify the DNA fragments, the following primers were used: Glyt_F577 and Glyt_R1562 (Li et al., 2007) for the nuclear gene glycosyltransferase 1 (*GLYT*); ENC1_F85, ENC1_R982, ENC1_F88 and ENC1_R975 (Li et al., 2007) for the nuclear gene ectodermal-neural cortex 1 (*ENC1*); Rh193F and Rh1039R (Chen et al., 2003) for the nuclear gene rhodopsin (*Rho*); LCO1490 (Folmer et al., 1994) and COX1R (Costa and Amorim, 2011) for the mitochondrial gene cytochrome c oxidase I (*Cox1*); 16sar-L and 16sbr-H (Palumbi et al., 2002) for the mitochondrial ribosomal gene 16s (*16s*). Polymerase chain reaction (PCR) was performed in 15 μl reaction mixtures containing 5× Green GoTaq Reaction Buffer (Promega), 3.2 mM MgCl2, 1 μM of each primer, 75 ng of total genomic DNA, 0.2 mM of each dNTP and 1U of Taq polymerase. The thermocycling profile was: (1) 1 cycle of 4 min at 94 °C; (2) 35 cycles of 1 min at 92 °C, 1 min at 47–59 °C (varying according the fragment) and 1 min at 72 °C; and (3) 1 cycle of 4 min at 72 °C. In all PCR reactions, negative controls without DNA were used to check contaminations. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing reactions were made using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 10 μl reaction volumes containing 1 μl BigDye 2.5, 1.55 μl 5× sequencing buffer (Applied Biosystems), 2 μl of the amplified products (10–40 ng), and 2 μl primer. The thermocycling profile was: (1) 35 cycles of 10 s at 96 °C, 5 s at 54 °C and 4 min at 60 °C. The sequencing reactions were purified and denatured and the samples were run on an ABI 3130 Genetic Analyzer. For each PCR product both strands were sequenced to generate the analyzed sequences. The program MEGA 6 (Tamura et al., 2013) were used to edit the sequences.

2.3. Phylogenetic analysis

Sequences of protein-coding genes were aligned using Clustal W (Chenna et al., 2003), after which the DNA sequences were translated into amino acids residues with MEGA 6.0 to test for the absence of premature stop codons or indels. The best fitting evolutionary model of each codon position of protein-coding sequences using Akaike information criteria (AIC) was determined with the software jModeltest version 2.1.7 (Darriba et al., 2012). We partitioned the dataset into seven partitions in the dataset containing only nuclear genes, and nine partitions when concatenating all nuclear and mitochondrial genes, according to the model found for each codon position of protein-coding sequences (Table S2). We performed phylogenetic analyses using Bayesian inference (BI) and maximum parsimony (MP) methods. BI analysis was conducted using MrBayes v3.2.5 (Ronquist et al., 2011) with the following settings: two Markov chain Monte Carlo (MCMC) runs of two chains each for 1 million generations, a sampling frequency of 100. All parameters between partitions except topology and branch lengths were unlinked. The appropriate burn-in fraction and convergence of the MCMC chains were graphically assessed by evaluating the stationary phase of the chains using Tracer v. 1.5 (Rambaut et al., 2013). The final consensus tree and Bayesian posterior probabilities (PP) were generated with the remaining tree samples after discarding the first 25% of samples as burn-in. The MP analysis was conducted with the software TNT 1.1 (Goloboff et al., 2008). Genetic information obtained in gene analysis of the sampled genes are shown in Table S3. We also performed a maximum likelihood (ML) analysis with the software Gali (Zwickl, 2006); since the resulting ML was identical to that found in other analysis, it