Molecular diagnosis of the arowanas *Osteoglossum ferreirai* Kanazawa, 1966 and *O. bicirrhossum* (Cuvier, 1829) from the Orinoco and Amazon River basins

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The arowanas, fishes of Gondwanan origin, are represented in South America by the genus *Osteoglossum*. All species were initially reported as being exclusive to the Amazon region, with *O. ferreirai* restricted to the Negro River basin and *O. bicirrhosum* to the Amazon and Essequibo Rivers basin. Starting in the mid 1970's it was reported that *O. ferreirai* also occurs in the Orinoco River basin. In all regions the arowanas assumed significant socio-economic importance due to their popularity in the international ornamental fish trade, leading to over-exploitation of both species in some areas. The Orinoco populations are particularly heavily exploited, and thus conservation and management measures are needed. Both depend on the clarification of taxonomic status, and phylogenetic distinctness of the Orinoco populations. With the goal of molecularly characterizing the two species of *Osteoglossum*, and comparing populations of *Osteoglossum* from the Orinoco and Amazon basins, we characterized individuals sampled from eight localities, one in the Orinoco River basin and seven in the Amazon River basin. We sampled 39 individuals, obtaining 1004 base pairs, of which 79 were synapomorphies. Genetic distance between the two species calculated using the HKY + G model of molecular evolution was 8.94%. Intraspecific distances ranged from 0.42% in *O. bicirrhosum* to 0.10% in *O. ferreirai*. The genetic characterization confirmed the taxonomic status of *O. ferreirai* in the Orinoco basin, and suggested that its distribution in the Orinoco basin is unlikely to be the result of vicariance or natural dispersal, but rather an anthropic introduction.

Os aruanãs, peixes de origem Gondwana, são representados na América do Sul pelo gênero Osteoglossum. Todas as espécies foram inicialmente reportadas como sendo exclusivamente da região Amazônica, com *O. ferreirai* restrito a bacia do Rio Negro e *O. bicirrhosum* para a bacia Amazônica e rio Essequibo. No meio dos anos 70 foi reportado que *O. bicirrhosum* também ocorre na bacia do rio Orinoco. Em todas as regiões os aruanãs são de significante importância socio-econômica devido a sua popularidade no comércio internacional de peixes ornamentais, levando a sobre-exploração de ambas as espécies em algumas áreas. As populações no rio Orinoco são particularmente muito exploradas, e assim medidas de conservação e manejo são necessárias. Ambas as medidas dependem de clarificações do estatus taxonômico, e distinções filogenéticas das populações do Orinoco. Com o objetivo de caracterizar molecularmente as duas espécies de *Osteoglossum*, e comparar as populações de *Osteoglossum* das bacias do Orinoco e Amazonas, nós caracterizamos indivíduos amostrados de oito localidades, uma da bacia do rio Orinoco e sete da bacia Amazônica. Nós amostramos 39 indivíduos, obtendo 1004 pares de bases, dos quais 97 foram sinapomorfias. A distância genética entre as duas espécies, calculadas usando-se o modelo HKY+G de evolução molecular foi de 8.94%. Distâncias intraespecíficas variaram de 0.42% em *O. bicirrhosum* a 0.10% em *O. ferreirai*. A caracterização genética confirmou o estatus taxonômico de *O. ferreirai* na bacia do rio Orinoco, e sugere que sua distribuição na bacia do rio Orinoco é improvavel de ser o resultado de um evento de vicariância ou dispersão natural, sendo melhor explicada como uma introdução antrópica.

Key words: Aruana, Biological introduction, Black arowana, Silver arowana.

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Introduction

The osteoglosids, known as the bony tongue fishes, are ancient creatures whose diversification is associated with the breakup of Gondwana (Carroll, 1988; Kumazawa & Nishida, 2000). Currently, ten species are known with distribution in the following regions: three in South America, one in Africa, four in Asia and two in Australia (Nelson, 2006). In South America, osteoglossids are represented by *Arapaima gigas* of the family Arapaimidae, and *Osteoglossum bicirrhosum* and *Osteoglossum ferreirai* of the family Osteoglossidae, distributed in the Amazonas, Essequibo and Orinoco basins (Reis, *et al.*, 2003; Watkins, *et al.*, 2004; Maldonado-Ocampo, *et al.*, 2008). The existence of arowanas in South America was reported first by the Portuguese naturalist Alexandre Rodriguez Ferreira during expeditions in the Brazilian Amazon between the years 1783 to 1792. Based on specimens he collected and his illustrations of the white arowana, Cuvier erected the genus and described the species *O. bicirrhosum*. It was Wallace during his 1845 expedition to the Negro River (Brazil) who found and illustrated for the first time the black arowana, however, this individual was lost during his return trip to England. The black arowana was described in 1966 by Kanazawa as *Osteoglossum ferreirai*, and its distribution was restricted to The Negro River basin, with rest of the Amazon and Essequibo basin being occupied by *O. bicirrhossum*. Based on an ichthyological survey performed by Cala (1973), the range of *O. ferreirai* was expanded to the lower Tomo River in the Colombian Llanos. By the turn of the millennium, *O. ferreirai* was being reported in the Bita, Tomo and Tuparro Vichada Rivers by Mojica (1999), Lasso *et al.* (2005) and Maldonado-Ocampo *et al.* (2006; 2008).

The arowanas from the Bita River appear to differ in their reproductive biology from those of the Negro River. A study of Gutiérrez *et al.* (2009) showed that the Bita River population had lower fecundity, producing 38 to 127 oocites per female and maturing at 62 cm TL, while in the Negro River populations females produced an average of 136 oocites, and matured sexually at 70 cm TL (Rabello-Neto, 1999; 2002). These differences were viewed by the authors as indication of population or even species level differences.

The two South American species of arowanas began to be exported in large numbers for the aquarium trade in the 1970's when they started to replace the Asian arowana *Scleropages formosus*, also known as dragon fish, after *S. formosus* was listed as endangered by CITES (Yue, *et al.*, 2003). Starting in the 1970's, over-exploitation of South American arowanas turned these species threatened with extinction in Colombia (Alvarez-León, 2002a; 2002b). In 2005, Traffic and WWF reunited prominent ichthyologists in the "International Workshop of ornamental fishes from northern South America". They discussed the high vulnerability of both *Osteoglossum* species, and proposed to include them in Appendix II of CITES. They considered as first action the necessity to clarify the taxonomic status of *O. ferreirai* in the Orinoco basin, its divergence from the Negro basin populations, and an assessment of genetic variability of *O. bicirrhossum* in its area of distribution.

Material and Methods

We obtained and analyzed fishes caught by artisanal fishermen at these locations: the Orinoco River basin in the Bita River (Puerto Careño, n = 5), the Amazon River basin: the Negro River (Barcelos, n = 7), the Putumayo River (Puerto Leguizamo, n = 8), the Juruá River (Eirunepé, n = 5), the Madeira River (Humaita, n = 4) and the Solimões-Amazonas (Tabatinga, n = 4; Tefé, n = 3; Santarém, n = 3) Fig.1.

All tissue samples were preserved in absolute alcohol and processed in the Laboratório de Evolução e Genética Animal (LEGAL) at the Universidade Federal do Amazonas (UFAM), Manaus, Brazil. DNA extraction was performed with the CTAB method (Doyle & Doyle, 1987), and quantified by means of electrophoresis in a 0.8% agarose gel against a known quantity of a molecular size standard (Fermentas).

Subsequently we amplified via the PCR (Polymerase Chain Reaction) a segment of mitochondrial DNA between the 3' end of cytochrome oxidase subunit 2 and the 5' end of the cytochrome oxidase subunit 3, including the tRNA Lysine, and ATPase subunits 6 and 8. This region was amplified using the primers L8106 5'-TGGGTGTTAAAATAGATGC-3' and H9264 5'-GAGGAGAGCRGCRGATGCCCC-3' and sequenced with L8106, L8537 5'-TGAAACTGACCATGACACTAAG-3' and H8516 5'-CTTGTGTCATGGTCAGTTTCA-3' developed by Hrbek *et al.* (2005) for *Arapaima gigas*.

The PCR had a total volume of 15 ìl, and included 5.6 µl of ddH$_2$O, 1.2 µl of 25 mM MgCl$_2$, 1.2 µl of 10X buffer (75 mM Tris HCl, 50 mM KCl, 20 mM (NH$_4$)$_2$SO$_4$), 1.5 µl of 10 mM dNTP, 1.5
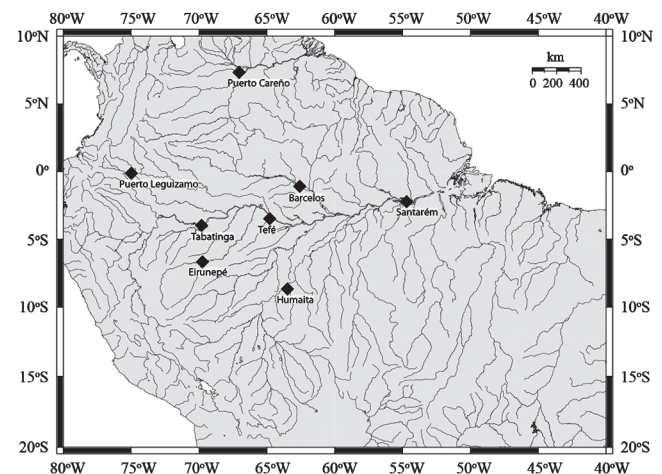

Fig. 1. Sampling localities in the Amazon and Orinoco basins of *O. bicirrhossum* and *O. ferreirai* . Base map was obtained from Online Map Creation (Geomar) currently available as Planiglobe (http://www.planiglobe.com/).

μl of each primer (2 μM), 1.0 μl nuclease-free BSA (2.5 mg / ml), 0.5 μl of Fermentas Taq DNA polymerase (1 U/μl) and 1.0 μl DNA (~20 ng/μl).

The PCR reactions were tested on 1% agarose gel. Sequencing reactions were performed using the BigDye Terminator Kit V.3.1. according to the manufacturer's guidelines and the product was analyzed on an ABI 3130xl automated sequencer. The sequences obtained were viewed and edited by using the program BioEdit (Hall, 1999). The dataset was aligned in Crustal W (implemented within Bioedit) and then adjusted manually (Thompson, *et al.*, 1996). The composition of nucleotide bases, identification of variable sites, stop codons, transversions/transitions and genetic distances was estimated in the program MEGA5 (Tamura, *et al.*, 2011). The number and frequency of haplotypes was determined in the program DnaSP v.5 (Librado & Rozas, 2009).

The program Modeltest 3.7 was used to test for the best molecular evolutionary model fitting the dataset (Posada & Crandall, 1998). Phylogenetic reconstruction was performed using the maximum likelihood method (ML) implemented in the program Treefinder (Jobb, *et al.*, 2004) and support for relationships was assessed via 1000 bootstrap pseudo-replications. We used *Scleropages formosus* and *Scleropages leichardti* as outgroups (Genbank No. DQ023143, FJ890319).

Phylogenetic species were identified using the Population Aggregation Analysis algorithm, that involves a search for fixed differences between local populations, followed by successive rounds of aggregation of populations and previously aggregated population groups that are not distinct from each other (Davis & Nixon, 1992), and complementary analytical procedures described by Cook *et al.* (2010).

Results

The final aligned sequences had a length of 1004 bp and 79 of which were diagnostic for the two species. The 79 synapomorphies were divided between 73 transitions and 6 transversions. The base pair composition of the data set was A = 27.2%, T = 29.1%, C = 30.2%, G = 13.4%. In total eleven haplotypes were identified for both species, seven in *O. bicirrhosum* and four in *O. ferreirai*. In both species a single haplotype was the most frequent (online Supplement 1-3). Haplotypes are available under the GenBank accession numbers JQ436740 to JQ436750.

The maximum likelihood topology using the HKY+G model of molecular evolution (Posada & Crandall, 1998) showed the formation of two evolutionary lineages representing the two species. The clade representing *O. bicirrhosum* split into two sub-clades. The first comprised the majority of individuals from the Putumayo (Puerto Leguizamo), Jurua (Eirunepé) and Solimões (Leticia) Rivers and the second was formed by majority of individuals in the Madeira (Humaitá), Solimões (Tefé) and Amazon (Santarém) Rivers. The clade representing *O. ferreirai* indicated the existence of a single evolutionary lineage consisting of individuals from the Bita (Orinoco basin) and from the Negro (Amazon basin) Rivers (Fig 2). Genetic

divergence between the two species of *Osteoglossum* averaged at 8.94%, while intraspecific divergence in *O. bicirrhosum* was 0.42% and *O. ferreirai* was 0.10% (Tables 1-2).

Population Aggregation Analysis (online Supplement) also resulted in the identification and diagnosis of two species:

Osteoglossum bicirrhosum (Cuvier, 1829)

DNA Diagnosis. This species can be distinguished from *O. ferreirai* by combinations of the following nucleotide characters: COII region: 528T, 561C, 567C, 582A, 585G, 603C, 606C, 612T, 618C, 648G. Lysine tRNA region: 22T, 41C, 45G. ATP8 region: 6A, 12C, 15T, 27G, 30A, 33G, 37T, 54G, 72C, 84C; 112A. ATP6 region: 87C, 96C, 111C, 114C, 138A, 150T, 192G, 198C, 213T, 217T, 219T, 226G, 249C, 258G, 265T, 267A, 282C, 285A, 294G, 300G, 312G, 313T, 318G, 324A, 330G, 333C, 339T, 351T, 363A, 393T, 394G, 410C, 429C, 447C, 448C, 450C, 474G, 489T, 495C, 508T, 528C, 552T, 576G, 583A, 585T, 586T, 591T, 600A, 607C, 625G, 627T, 633A, 654G, 657A.

Morphological features (data from Kanazawa, 1966). Tip of snout to anus 45.5 to 53.2; snout to origin of dorsal fin 55.9 to 61.9; snout length 3.7 to 6.5; eye diameter 3.1 to 8.7; head length 19.6 to 26.9; body depth 14.6 to 20.7. These data are given in percentages with respect to standard length. Branched dorsal fin rays 42 to 50; unbranched anal fin rays 49 to 58; pectoral rays i, 6; scales along the lateral line to base of caudal fin 30 to 37; pre-dorsal scales 16 to 19. Adult color is varied and can be silver, yellow, or dark greenish, paler ventrally.

Distribution. Essequibo River basin; Amazon River basin not including the Negro River basin except its Branco River affluent.

Osteoglossum ferreirai Kanazawa, 1966

DNA Diagnosis. This species can be distinguished from *O. bicirrhosum* combinations of the following nucleotide characters: COII region: 528C, 561T, 567T, 582G, 585A, 603T, 606T, 612C, 618T, 648A. Lysine tRNA region: 22C, 41T, 45A. ATP8 region: 6G, 12T, 15C, 27A, 30G, 33A, 37C, 54A, 72T, 84T; 112G. ATP6 region: 87T, 96C, 111T, 114T, 138G, 150C, 192A, 198T, 213C, 217C, 219A, 226A, 249T, 258A, 265C, 267G, 282T, 285C, 294A, 300A, 312A, 313C, 318A, 324C, 330A, 333T, 339C, 351G, 363G, 393A, 394A, 410T, 429T, 447T, 448T, 450A, 474T, 489C, 495T, 508C, 528T, 552C, 576A, 583G, 585C, 586C, 591C, 600G, 607T, 625A, 627C, 633G, 654A, 657G.

Morphological features (data from Kanazawa, 1966). Tip of snout to anus 48.5; snout to origin of dorsal fin 53.6; snout length 6.4; eye diameter 7.0; head length 22.6; body depth 13.9. These data are given in percentages with respect to standard length. Branched dorsal fin rays 52 to 57; unbranched anal fin rays 61 to 66; pectoral rays i, 6; scales along the lateral line to base of caudal fin 37 to 40; pre-dorsal scales 15 to 19. Adult color is dark bluish, paler ventrally.
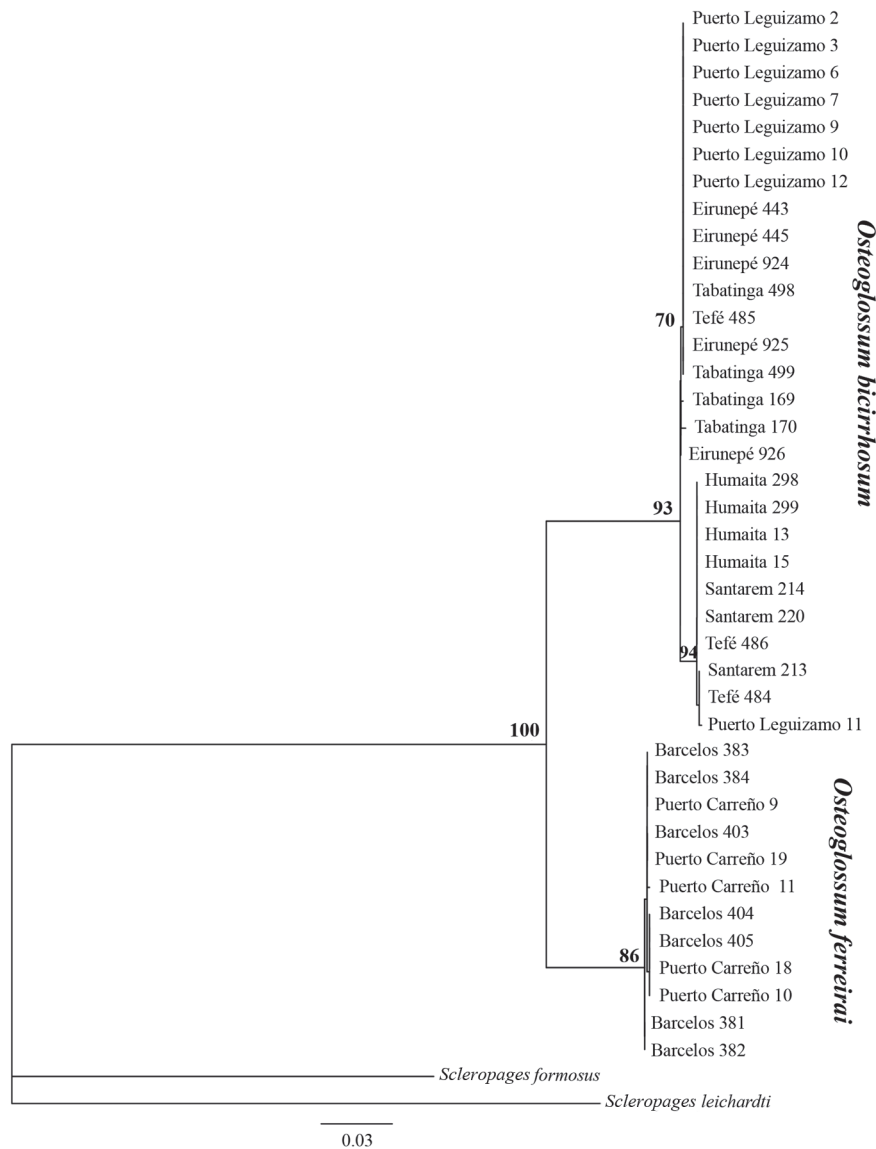
Puerto Leguizamo 2
Puerto Leguizamo 3
Puerto Leguizamo 6
Puerto Leguizamo 7
Puerto Leguizamo 9
Puerto Leguizamo 10
Puerto Leguizamo 12
Eirunepé 443
Eirunepé 445
Eirunepé 924
Tabatinga 498
Tefé 485
Eirunepé 925
Tabatinga 499
Tabatinga 169
Tabatinga 170
Eirunepé 926
Humaita 298
Humaita 299
Humaita 13
Humaita 15
Santarem 214
Santarem 220
Tefé 486
Santarem 213
Tefé 484
Puerto Leguizamo 11

Osteoglossum bicirrhosum

70
93
94
100

Barcelos 383
Barcelos 384
Puerto Carreño 9
Barcelos 403
Puerto Carreño 19
Puerto Carreño 11
Barcelos 404
Barcelos 405
Puerto Carreño 18
Puerto Carreño 10
Barcelos 381
Barcelos 382

Osteoglossum ferreirai

86

Scleropages formosus

Scleropages leichardti

0.03

Fig. 2. Maximum likelihood phylogenetic hypothesis of relationships of *Osteoglossum* individuals representing the species *O. bicirrhossum* and *O. ferreirai*.

Distribution: Negro River basin, including the affluent Branco River; Orinoco River basin.

Discussion

Phylogenetic and Population Aggregation Analysis clearly separate and diagnose the two species of *Osteoglossum*, and place the Colombian populations within the species *O. ferreirai*. This diagnosis confirms that of Cala (1973). There were no unique haplotypes in the Orinoco basin samples of *O. ferreirai*, indicating a very recent divergence of the populations of the Orinoco and Negro River basins. We suggest that the lack of divergence of the Orinoco basin samples is most likely due to a recent introduction from the Negro to the Orinoco river basin. Based on ichthyofaunal surveys of areas that can potentially serve as biological corridors and links between the two basins such as the Casiquiare channel and the floodplain of the Inirida and Atabapo Rivers, neither *O. ferreirai* nor *O. bicirrhossum* is

Table 1. Table of K2P distances (Kimura, 1980) between the two species of *Osteoglossum* and the Negro and Orinoco River populations of *O. ferreirai*.

	O. bicirrhossum	*O. ferreirai* Negro
O. ferreirai Negro	0.0892	
O. ferreirai Orinoco	0.0897	0.0009

Table 2. Table of intraspecific and intragroup K2P distances (Kimura, 1980) observed in the two species of *Osteoglossum* and the Negro and Orinoco River populations of *O. ferreirai*.

O. bicirrhossum	0.0042
O. ferreirai Negro	0.0010
O. ferreirai Orinoco	0.0010

present in these areas (Maldonado-Ocampo, *et al.*, 2006; Maldonado-Ocampo, *et al.*, 2008; Winemiller *et al.*, 2008; Lasso, *et al.*, 2009; Miller-Hurtado*, et al.*, 2009; Winemiller & Willis, 2011). Additionally, based on fauna surveys (Cala, 1973; Mojica, 1999; Lasso *et al.*, 2005; Maldonado-Ocampo *et al.*, 2006; 2008) the expansion in the distribution of *O. ferreirai* in Colombia appears to have proceeded from north to south, i.e. towards the Negro River basin. Therefore, we consider it highly unlikely that the occupation of the Orinoco basin would proceed via geographically intermediate areas between the Orinoco and Negro basins. Natural long distance dispersal and colonization also appears an unlikely mechanism. Although we could not find any reference or report that would suggest that *O. ferreirai* in the Orinoco basin was or could have been introduced, we consider an introduction the most likely explanation. The area of origin of *O. ferreirai* from the Orinoco basin is unclear, however, and will require a detailed characterization of *O. ferreirai* from the Negro basin.

The possibility and what we consider to be the most likely scenario that *O. ferreirai* has been introduced in the Orinoco basin, constitutes a management and conservation dilemma. The Orinoco *O. ferreirai* have genetic diversity comparable to that of the fishes found in the Negro River basin, and appear to have expanded their geographic distribution. Thus they appear likely to increase in density and expand geographically if an ecological opportunity presents itself. On one hand, *O. ferreirai* makes an important contribution to the local economy, and is a highly desirable export species in the aquarium trade. In the Orinoco basin it appears to be over-exploited (Alvarez-León, 2002b; CCI, 2009), hence implementing a management and/or conservation program may result in not only its recovery, but also its expansion to other areas where it is currently not found. Being a top of the pyramid predator, and predating not only aquatic but also terrestrial vertebrates, could have a major impact on population ecology of the areas where densities *O. ferreirai* will increase. On the other hand, conservation programs normally try to eliminate introduced species. However, it seems unlikely that a program to eradicate *O. ferreirai* would succeed due to the area already colonized, and the remoteness of these areas. A third option is to do nothing. Under this scenario, the global aquarium market will largely determine if the species increases in density and colonizes new areas, remains at *status quo*, or its densities will decrease and possibly will even experience local extinctions. Ultimately, however, conservation and management decisions will be mandated by governmental agencies and will likely tend to balance socioeconomic needs with environmental protection.

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