Gene 511 (2012) 320–325

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short Communication

Mitochondrial DNA as effective molecular markers for the genetic variation and phylogeny of the family Osteoglossidae

Xi-dong Mu, Xue-jie Wang, Hong-mei Song, Ye-xin Yang, Du Luo, Dang-en Gu, Meng Xu, Chao Liu, Jian-ren Luo, Yin-chang Hu *

Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Key Laboratory of Tropical & Subtropical Fishery Resource Application & Cultivation, Ministry of Agriculture, Guangzhou 510380, China

ARTICLE INFO

Article history:
Accepted 12 September 2012
Available online 2 October 2012

Keywords:
Osteoglossidae
Phylogenetic analysis
Species identification

ABSTRACT

The present study examined the genetic variation of the family Osteoglossidae from different geographical locations based on the mitochondrial NADH dehydrogenase subunit 2 (ND2) and *ATPase* subunit 6 (*ATPase*6) genes; we then re-constructed the phylogenetic relationships using the two sequences in combination. The results showed that the partial sequences of mitochondrial ND2 and *ATPase*6 of the family Osteoglossidae were 813 bp and 669 bp, respectively. A total of 42 species-specific nucleotide positions of the family Osteoglossidae were found to be useful for molecular identification. The sequence variation showed greater differences (8.3%~28.1% for the combined sequences, 8.3%~26.7% for the ND2 gene, and 9.3%~28.7% for the *ATPase*6 gene) among the different species of Osteoglossidae, and there was a significant association between the genetic difference and geographical location. Phylogenetic analyses using neighbor-joining, Bayesian inference, and maximum parsimony (MP) methods based on the combined sequences of the two genes were able to distinguish the different species and were in agreement with the existing taxonomy based on morphological characters and in association with the geographical distribution among seven species of the family Osteoglossidae.

1. Introduction

The family Osteoglossidae (bonytongues) is a relatively primitive teleostean clade with a significance for evolutionary biology that far outweighs their relatively modest diversity, which is restricted to freshwater habitats (mostly tropical or subtropical) on the continents of Gondwanian origin, including Asia, the America and Africa (Nelson, 2005; Wilson and Murray, 2008). According to the currently accepted taxonomy based on morphological characters, the family Osteoglossidae includes two extant subfamilies, Heterotidinae and Osteoglossinae. The former comprises two species: *Heterotis niloticus* (Nile arowana) of Western Africa, characterized as microphagous (Taverne et al., 2007; Ugwumba, 1993), and *Arapaima gigas* (pirarucu) of South America, one of the largest scaled freshwater fish species (Debora et al., 2006). The latter subfamily includes five species: *Osteoglossum bicirrhosum* (silver arowana) and *O. ferreirai* (black arowana) of South America; *Scleropages jardinii* (northern barramundi) of Northern Australia and New Guinea; *S. leichardti* (spotted barramundi) of Eastern Australia; and *S. formosus* (Asian arowana) distributed in Southeast Asia. *S. formosus* is the most valuable ornamental fish that is listed by the Convention on International Trades in Endangered Species of Wild Fauna and Flora (CITES) as a highly endangered species; this species is threatened with extinction in its native localities because of overfishing (or illegal fishing) and trading (Greenwood et al., 1966; Tang et al., 2004; Yue et al., 2002, 2006). The unique anatomy of this fish has created interest in its early morphology and behavior. In addition, the geographic distribution of *S. formosus* in the fresh waters of five biogeographical regions has been cited as evidence for its dispersal from a single origin (Wilson and Murray, 2008). Due to the great ecological value of the extant Osteoglossidae inhabiting terrestrial regions mostly of Gondwanian origin (Kumazawa and Mutsumi, 2000), the accurate phylogenetic and biogeographical relationships should be determined because it has important implications for studying the evolution of freshwater fishes and biogeography.

A number of DNA-based molecular techniques, including simple sequence repeat (SSR) (Mu et al., 2011; Shafiqur et al., 2008), random amplified polymorphic DNA (RAPD) (Yue et al., 2002), amplified fragment length polymorphism (AFLP) (Yue et al., 2004) and mitochondrial DNA (Kumazawa and Mutsumi, 2000; Tang et al., 2004), have been used to establish the population and genetic relationships of some individuals or a particular group of related individuals of certain Osteoglossidae species, such as *S. formosus* (Mohd-Shamsudin et al., 2011; Mu et al., 2011; Tang et al., 2004), *A. gigas* (Tomas and Izeni,

Abbreviations: mtDNA, mitochondrial DNA; ND2, NADH dehydrogenase subunit 2; *ATPase*6, *ATPase* subunit 6; PCR, Polymerase Chain Reaction; NJ, neighbor-joining; MP, maximum parsimony.

* Corresponding author at: Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, 1 Xingyu Road, Liwan District, Guangzhou City, Guangdong Province, China. Tel.: +86 20 81616088; fax: +86 20 81616162(work).
E-mail address: huyc22@163.com (Y. Hu).

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.09.087

2008) and *O. bicirrhosum* (Inoue et al., 2001). However, aside from these studies, there have been few scientific reports on the inter- or intra-species identification and phylogenetics among the family Osteoglossidae. The lack of information on the phylogenetic relationships has hindered efforts to illustrate the reason for the geographic dispersal of Osteoglossidae species.

Mitochondrial (mt) DNA provides useful molecular markers for studies on problematic identification and phylogeny (Avise, 2004). It was reported that biologically distinct geographical individuals or populations of different Osteoglossidae species showed high variation in their mitochondrial NADH dehydrogenase subunit 2 (ND2) (Kumazawa and Mutsumi, 2000) and *ATPase* subunit 6 and 8 (*ATPase* 6 and 8) genes (Tang et al., 2004). Therefore, these genes should provide better markers for phylogenetic and biogeographical studies.

The objective of the present study was to compare the sequence variability of the mitochondrial ND2 and *ATPase*6 genes and to examine the inter- and intra-species relationships of the family Osteoglossidae. The phylogenetic relationships of Osteoglossidae were re-constructed based on the combined sequences of these two mtDNA genes.

2. Materials and methods

2.1. Taxonomic sampling and isolation of genomic DNA

A total of 69 Osteoglossidae samples have been used in this analysis. A portion of the samples was collected from several farms between 2005 and 2011, whereas other samples were legally imported from Malaysia and maintained in our lab. The species, sample code, country of origin, and their GenBank accession numbers are listed in Table 1. All fin clips were scissored tail fin with narcosis, fixed in 95% ethanol and stored at −20 °C. The total genomic DNA was extracted using Tissue DNA Kit (OMEGA E.Z.N.A) according to the manufacturer's protocol.

2.2. PCR protocols and sequencing

The PCR reactions (25 μl) were performed in 2 mM $MgCl_2$, 2.5 μM each primer, 2.5 μl 10× PCR buffer, 0.2 μM each dNTP, 1.25 U *Taq* DNA polymerase (TAKARA), and 1 μl DNA sample. The mitochondrial ND2 was amplified using primers lyND2-F (5′-CAGCTC MCACTGAYTHCTAG-3′) and lyND2-R (5′-GWGGWGTAGCAT ARGCGTAG-3′) under the following conditions: an initial denaturation at 94 °C for 4 min; 33 cycles of 94 °C for 30 s (denaturation), 56 °C for 30 s (annealing), and 72 °C for 45 s (extension); followed by a final extension at 72 °C for 6 min. The partial *ATPase*6 gene was amplified using primers lyatp6-F (5′-ATGACAC TAAGCTTYTTYGAYC-3′) and lyatp6-R (5′-TTAWACGGATTCTTGTAARTAAAG-3′) under the following conditions: an initial denaturation at 94 °C for 4 min; 33 cycles of 94 °C for 1 min (denaturation), 50 °C for 30 s (annealing), and 72 °C for 45 s (extension); followed by a final extension at 72 °C for 8 min. These optimized cycling conditions for the specific and efficient amplification of the individual mtDNA fragments were obtained after varying the annealing temperatures. The PCR products were examined using 1% agarose gel electrophoresis to validate the amplification efficiency. The positive amplicons were selected, purified and sequenced using an ABI 377 automated DNA sequencer employing the same primers (individually) as used in the PCR.

2.3. Phylogenetic analyses

The sequences of the mtDNA ND2 and *ATPase*6 genes for 69 Osteoglossidae samples were aligned separately using Clustal X 1.83 (Thompson et al., 1997) with the default parameters, and the alignments were exported to Seaview (Galtier et al., 1996) to be examined and adjusted by hand, as required. The sequence inter-species and intra-species divergence of the family Osteoglossidae was evaluated based on the single or combined sequences using Mega5.05 (Tamura et al., 2011).

We used a combination of the two sequence fragments for inferring the phylogenetic relationships among all of the species because many published studies show that multiple sequence data sets can be combined when incongruence is detected (Wu et al., 2005). The best-fit model of sequence evolution was determined using jModeltest 0.1.1 (Posada, 2008), selecting the AIC model. The phylogenetic trees were constructed using the neighbor-joining (NJ), Bayesian inference (BI), and maximum parsimony (MP) methods. The NJ analysis was performed using MEGA 5.05, and the BI analysis was performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). Both runs began with a random starting tree and ran for 2×10^7 generations, sampling the trees every 100 generations. The standard deviation of the split frequencies for the combined sequences was well below 0.01, indicating that the number of generations was sufficient. The MP analysis was performed using TBR branch swapping and 10 random taxon addition replicates under a heuristic search, saving no more than 100 equally parsimonious trees per replicate in PAUP*4.0 b10 (Swofford, 2002). To study genetic relationships of the family Osteoglossidae, *Notopterus notopterus*

Table 1
Species, sample number (codes), origins of the members, the G content, number of haplotype, and GenBank accession numbers for the partial sequences of the mitochondrial NADH dehydrogenase subunit 2 (mtDNA ND2) and *ATPase* subunit 6 (mtDNA *ATPase*6) genes of the family Osteoglossidae used in this study.

Species		Sample number (sample code)	Country of origin	mtDNA ND2 gene			mtDNA *ATPase*6 gene		
				G content (G %)	Number. of haplotype	GenBank accession no.	G content (G %)	Number. of haplotype	GenBank accession no.
Osteoglossum bicirrhosum		9(OBYL1-OBYL 9)	Amazon basin, South America	11.2	9	JQ337773–JQ337781	11.2	8	JQ337814–JQ337821
Osteoglossum ferreirai		6(OFHL1-OFHL6)	Negro River basin, South America	11.2	5	JQ337782–JQ337786	14.0	6	JQ337822–JQ337827
Scleropages formosus	Red arowana	21(SFHL1-SFHL 21)	Kalimantan, Indonesia, Asia	12.5	22	JQ337787–JQ337808	12.4	25	JQ337833–JQ337856
	Golden arowana	12(SFJL1-SFJL 10; SFGBJL1-SFGBJL 2)	Bukit MerahLake, Perak, Malaysia, Asia	12.5			12.1		
	Green arowana	5(SFQL1-SFQL 5)	Terengganu,Malaysia, Asia	12.6			11.9		
Scleropages leichardti		6(SLZZL1-SLZZL 6)	MacKenzie river, Eastern Australia	12.3	2	JQ337810–JQ337811	13.9	2	JQ337828–JQ337829
Scleropages jardinii		5(SJXZL1-SJXZL 5)	Gulf of carpentaria division, Northern Australia	14.8	2	JQ337812–JQ337813	14.0	3	JQ337830–JQ337832
Arapaima gigas		5(AGHX1-AGHX 5)	Amazon river, Brazil	10.5	3	JQ337807–JQ337809	10.9	3	JQ337856–JQ337858