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Molecular and morphological approaches for species delimitation and hybridization investigations of two *Cichla* species

Andrea A. F. Mourão[1], Diogo Freitas-Souza[2], Diogo T. Hashimoto[3], Daniela C. Ferreira[4], Fernanda D. do Prado[5], Rosicleire V. Silveira[5], Fausto Foresti[2] & Fábio Porto-Foresti[1]

1. Departamento de Ciências Biológicas, Faculdade de Ciências, Universidade Estadual Paulista (UNESP), Av. Eng. Luiz Edmundo Carrijo Coube, 14-01, Vargem Limpa, 17033-360 Bauru, SP, Brazil. (mouraoaaf@ibb.unesp.br; fpforesti@fc.unesp.br)
2. Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Distrito de Rubião Junior, s/n, Distrito de Rubião Junior, 18618-970 Botucatu, SP, Brazil. (souza.d.freitas@gmail.com; fforesti@ibb.unesp.br)
3. Centro de Aquicultura da UNESP, Campus de Jaboticabal, Universidade Estadual Paulista (UNESP), via de Acesso Prof. Paulo Donato Castellane, s/n, Vila Industrial, 14884-900 Jaboticabal, SP, Brazil. (diogo@caunesp.unesp.br)
4. Departamento de Biologia e Zoologia, Instituto de Biociências, Universidade Federal de Mato Grosso (UFMT), Av. Fernando Corrêa da Costa, 2367, Boa Esperança, 78060-900 Cuiabá, MT, Brazil. (ferreiradc@gmail.com)
5. Departamento de Biologia e Zootecnia, Campus de Ilha Solteira, Universidade Estadual (UNESP), Rua Monção, 226, Zona Norte, 15385-000 Ilha Solteira, SP, Brazil. (rosiverissimo@bio.feis.unesp.br)

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ABSTRACT. The hybridization is a widely-discussed issue in several studies with fish species. For some authors, hybridization may be related with diversification and speciation of several groups, or also with the extinction of populations or species. Difficulties to differentiate species and hybrids may be a problem to correctly apply a management of wild species, because hybrid lineages, especially the advanced ones, may resemble the parental species. The genus *Cichla* Bloch & Schneider, 1801 constitutes an interesting experimental model, considering that hybridization and taxonomic uncertainties hinder a correct identification. Considering these problems, in this study, we developed genetic methodologies and applied meristic and morphometric approaches in wild samples in order to identify species and for test a possible hybridization between *Cichla kelberi* Kullander & Ferreira, 2006 and *Cichla piquiti* Kullander & Ferreira, 2006. For this, *C. kelberi*, *C. piquiti* and potential hybrid (*carijó*) individuals were collected in Paraná and Tietê rivers (SP, Brazil). For meristic and morphometric methods, the individuals were analyzed using the statistical software Pcord 5:31, while for molecular methods, primers for PCR-multiplex were designed and enzyme for PCR-RFLP were selected, under the species-specific nucleotide. All results indicated that the *carijó* is not an interspecific hybrid, because it presented identical genetic pattern and morphology closed to *C. piquiti*. Thus, we propose that *carijó* is a *C. piquiti* morphotype. In addition, this study promotes a new molecular tool that could be used in future research, monitoring and management programs of the genus *Cichla*.

KEYWORDS. *Cichla kelberi*; *Cichla piquiti*; carijó; Cluster Similarity; PCA; PCR-multiplex.

RESUMO. Abordagens morfológicas e moleculares para delimitação de espécie e investigações de hibridação de duas espécies de *Cichla*. A hibridação é uma questão amplamente discutida em vários estudos com espécies de peixes. Para alguns autores, hibridações podem estar relacionadas à diversificação e especiação de muitos grupos, ou à extinção de populações ou espécies. Dificuldades para diferenciar espécies e híbridos podem ser um problema para aplicar corretamente o manejo de espécies selvagens, porque linhagens híbridas, especialmente as mais avançadas, podem assemelhar-se aos parentais. O gênero *Cichla* Bloch & Schneider, 1801 constitui um interessante modelo experimental, considerando que a hibridação e as incertezas taxonômicas dificultam a correta identificação. Considerando estes problemas, neste estudo foram desenvolvidas metodologias genéticas e aplicadas abordagens merísticas e morfométricas em amostras selvagens para identificar espécies e para testar uma possível hibridação entre *Cichla kelberi* Kullander & Ferreira, 2006 e *Cichla piquiti* Kullander & Ferreira, 2006. Para isto, *C. kelberi*, *C. piquiti* e indivíduos do híbrido em potencial (*carijó*) foram coletados nos rios Paraná e Tietê (SP, Brasil). Para os métodos merístico e morfométrico, os indivíduos foram analisados, utilizando-se o software estatístico Pcord 5:31, enquanto que para os métodos moleculares, primers para PCR-multiplex foram desenhados e enzimas para PCR-RFLP foram selecionadas, sob nucleotídeos espécie-específicos. Todos os resultados indicaram que o *carijó* não é um híbrido interespecífico, porque apresentou padrão genético idêntico e morfologia próxima à *C. piquiti*. Assim, propomos que o *carijó* é um morfotipo de *C. piquiti*. Além disso, este estudo promove uma nova ferramenta molecular que poderá ser utilizada em futuras pesquisas, monitoramento e manejo do gênero *Cichla*.

PALAVRAS-CHAVE. *Cichla kelberi*; *Cichla piquiti*; carijó; Análise de agrupamento; PCA; PCR-multiplex.

The most influent species concept was proposed by Mayr (1942, 1963) (Hausdorf, 2011). This concept defines species as a group of individuals or a population with potential to interbreed that is reproductively isolated from other groups. However, in several cases this concept is not applicable, especially for fish species. This is because studies have been demonstrated that hybridization might occur between specimens classified as distinct species, producing fertile progeny (Hashimoto *et al*., 2012; Prado *et al*., 2011, 2012). Conceivably, the hybridization is a widely-discussed issue because it questions fundamental biological concepts, such as species definition.

For the genus *Cichla* Bloch & Schneider, 1801 the hybridization is a natural phenomenon that could be related with diversification and speciation (Willis *et al*., 2012). On the other hand, for Toledo-Filho *et al*. (1998) and Millar *et al*. (2012) introgressive hybridization may also cause gene flow between different species or lineages, and in some cases, lead populations or species to extinction (Allendorf *et al*., 2001). In any case, difficulties to clearly differentiate hybridization could be a problem in monitoring wild species (Allendorf *et al*., 2001). This occurs because hybrids, especially the more advanced (e.g. resulting from introgression) may be morphologically similar to the parental (Allendorf *et al*., 2001). Nevertheless, studies commonly perform identification of hybrids through meristic and morphological characteristics without analyzing genetic markers (Scribner *et al*., 2001). However, the use of a unique source of analysis may lead to uncertain results (Chevassus, 1983; Mallet, 2005).

The hybridization for the genus *Cichla* in natural environment has been hypothesized by numerous studies (e.g. Alves & Feldberg, 1998; Brinn *et al*., 2004; Oliveira *et al*., 2006). Specifically, the hybridization between *Cichla kelberi* Kullander & Ferreira, 2006 and *Cichla piquiti* Kullander & Ferreira, 2006 has already been analyzed using genetic approaches as SPAR technique (Almeida-Ferreira *et al*., 2011) and the 5S rRNA marker (Oliveira *et al*., 2008). However, considering the limitations of these studies, such as the ambiguous results of the methodology SPAR and the problems to obtain species-specific markers reported by Oliveira *et al*. (2008), not confirming the presence of hybridization, it is necessary more studies for *C. piquiti* and *C. kelberi* and the supposed interspecific hybridization.

In addition to the hybridization problems there are taxonomic issues for the genus *Cichla*. According to Kullander & Ferreira (2006) identification key, *Cichla* has 15 valid species. For these authors, *Cichla kelberi* is frequently identified as *Cichla* **cf.** *monoculus*, while *C. piquiti* may be misidentified as *Cichla* sp. However, Willis *et al*. (2012) analyzed multi-locus data and questioned the validity of the Kullander & Ferreira (2006) identification key, rearranging these 15 species in eight evolutionarily significant units (ESUs). They also suggested *C. kelberi* and *C. ocellaris* synonymization as subspecies or distinct ESUs.

An aggravating factor for the hybridization within the genus and uncertainties concerning to taxonomic is the frequent introductions of species (Agostinho *et al*., 2007). According to these authors, species of *Cichla* are appreciated in sport (Kullander, 2003) and professional fishing (Sampaio *et al*., 2012), and are also commonly introduced in non-native environments to control other invasive species (Thresher *et al*., 2014). Due to this, *Cichla* species, naturally restricted to the Amazon basin (Kullander & Ferreira, 2006), have been frequently reported in several Brazilian basins (Pelicice & Agostinho, 2009).

These introductions of fish, even when aiming to control invasive species, generate disturbances in several trophic levels (Godinho *et al*., 1994; Latini & Petrere Jr, 2004). In general, the native ichthyofauna is affected with the presence of exotic piscivorous species, such as *Cichla*. For example, some studies reported a reduction or even disappearance of some species (Godinho *et al*., 1994; Latini & Petrere Jr, 2004).

A problem with the introduction of species is the high proliferation and phenotypic plasticity regulated with the environment-genotype interaction (Falconer & Mackay, 1996). This fact is expected for the genus *Cichla*, commonly known as *peacock bass*, due to the adaptive capacity to different environments (Carvalho *et al*., 2014). Thus, *Cichla* species constitute interesting experimental models to studies of ecology, genetic, toxicology, parasitology and, principally taxonomy currently (Kullander & Ferreira, 2006; Willis *et al*., 2012).

Studies that provide accessible and clear molecular markers for species identification are extremely important for species conservation (Young *et al*., 2001). Comparative molecular markers based on the detection of mtDNA (mitochondrial DNA) and nDNA (nuclear DNA) variability and diagnostic differences may enable to identify species based on variations even in a unique nucleotide polymorphism (Hashimoto *et al*., 2012), which would be taxonomically important for the group. The use of mtDNA and nDNA is important because hybrid individuals inherit nDNA from both parental but they inherit mtDNA just from maternal parental. The use of both nDNA and mtDNA markers allows to obtain a greater amount of information for species and hybrids identification (Aboim *et al*., 2010; Hashimoto *et al*., 2010; De-Franco *et al*., 2012; Gomes *et al*., 2012; Karlsson *et al*., 2012).

Considering hybridization, taxonomic problems and the frequent introductions as an aggravating factor in the genus *Cichla*, the development of a reliable methodology for hybrid identification is essential to assist to elucidate species identification and also to investigate the possibility of hybridization in wild stocks. In this study, we developed PCR-RFLP and PCR-multiplex methodologies based on mtDNA and nDNA for species identification. We also applied these genetic markers, meristic and morphometric analyses in wild samples in order to identify putative hybridization between *C. kelberi* and *C. piquiti*.

MATERIAL AND METHODS

A total of 125 individuals (both sexes, juveniles and adults) of the species *C. kelberi* (yellow *peacock bass*), *C. piquiti* (blue *peacock bass*) and individuals classified as putative hybrids of these two *Cichla* species (presenting a dubious external morphological identification, possible hybrids between the two-former species) were collected in Ilha Solteira (89 specimens), Rubineia (20 specimens), Pauliceia (10) and Uru (6 specimens) reservoirs in Paraná and Tietê rivers, São Paulo state, throughout the year 2009 (Fig. 1). From Ilha Solteira, 30 specimens were collected and used for meristic, morphometric and genetic analyses. Additionally, 95 specimens from Uru, Pauliceia e Rubineia

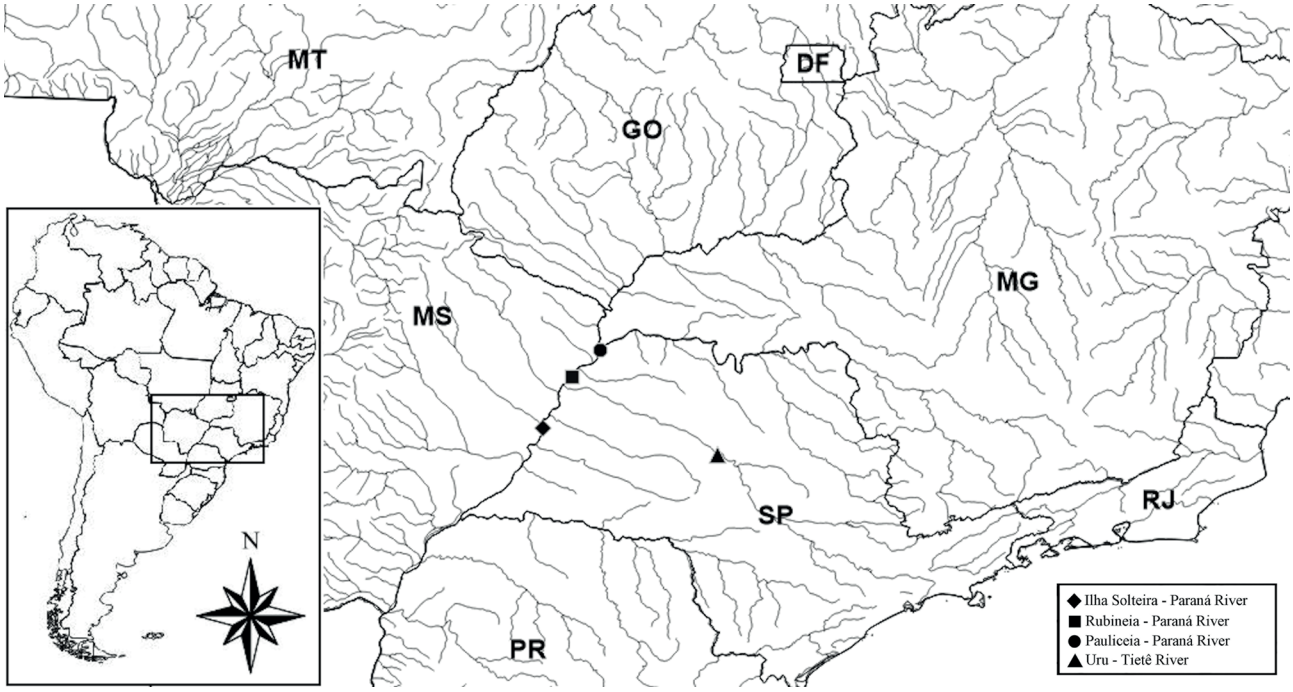
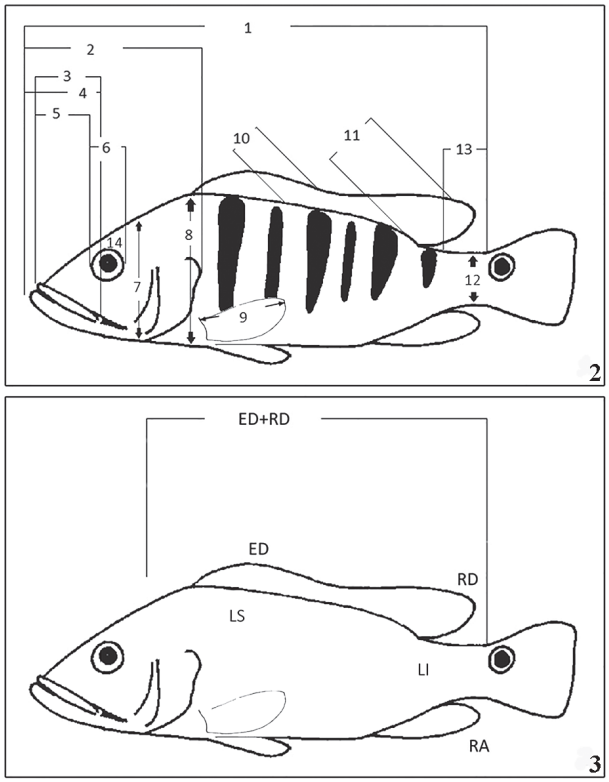


Fig. 1. Sample sites location in the Paraná and Tietê rivers, state of São Paulo, Brazil.

were collected and used for genetic analysis. The possible hybrids between *C. kelberi* and *C. piquiti* are hereafter referred as *carijó*. All analyses were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. The individuals meristic and morphometrically analyzed are deposited at the Laboratório de Ictiologia, Departamento de Zoologia e Botânica (UNESP, campus São José do Rio Preto), under the voucher numbers DZSJRP 8833, 8843, 8872 and 8946. The fin tissue samples genetically analyzed are deposited at the Laboratório de Genética de Peixes, (UNESP, campus Bauru).

For meristic and morphometric analyses, 30 specimens from Ilha Solteira were selected and analyzed in the Laboratório de Ictiologia Neotropical, according to Kullander (1986), Kullander & Nijssen (1989) and Kullander & Ferreira (2006) (Figs 2, 3). Body measurements were taken from point to point with a digital caliper (accuracy 0.01). Several colour characters were considered: number of vertical bars and the presence of post orbital and occipital bars, as proposed by Kullander & Ferreira (2006) (Figs 4-6). Morphometric data and their proportions (standard length and head length) were ordered using PCA. Meristic data were analyzed with a Cluster Similarity analysis (Euclidean distance). Both analyses were conducted in the statistical software Pcord 5:31 (Mccune & Mefford, 2006).

For the genetic analysis, 125 fin tissues from all the collection sites Ilha Solteira, Rubineia, Pauliceia and Uru were sampled and DNA extractions were performed according to the commercial protocol Wizard Genomic DNA Purification Kit (Promega). DNA quantification was tested for all samples using the Qubit 3.0 Fluorometer equipment.



Figs. 2, 3. Fig. 2 (adapted of Kullander & Ferreira, 2006), body measures used for morphological analyses of *Cichla* Bloch & Schneider, 1801 individuals: Standard length (1); head length (2); snout length (3); length of the lower jaw (4); length of the upper jaw (5); orbit diameter (6); head height (7); body height (8); length of pectoral fin (9); length of the dorsal fin spines (10); length of the dorsal fin radius (11); length of caudal peduncle (12); caudal peduncle height (13); and inter-orbital width (14). Fig. 3 (adapted of Kullander & Nijssen 1989): meristic parameters included the following measures. Numbers of dorsal fin spines (ED); dorsal fin radius (RD); upper lateral line scales (LS); lower lateral line scales (LI); pectoral fin radius (RP); and anal fin radius (RA).

For PCR-multiplex (Polymerase Chain Reaction-multiplex) and PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) reactions development, the cytochrome c oxidase universal subunit I (COI) and the recombination activating 1 (RAG1) genes were amplified under the PCR conditions described (Tab. I) with the primers described (Tab. II).

In order to design species-specific primers and to assign species-specific restriction enzymes we sequenced the mtDNA gene COI and the nDNA gene RAG1 using the Automatic Sequencer ABI3130 Capillary (Perkin-Elmer). In total, 43 amplification products (11 COI gene sequences - 5 *C. piquiti*, 3 *C. kelberi* and 3 *carijó* and 32 RAG1 gene sequences - 11 *C. piquiti*, 13 *C. kelberi* and 8 *carijó*; assorted collection sites) were analyzed. Both genetic markers, primers for PCR-multiplex were designed and enzyme for PCR-RFLP were selected, under the species-specific nucleotide differentiation. For this, the sequences were aligned using the ClustalW parameter (Thompson *et al*., 1994) implemented in the BioEdit software (Hall, 1999). Consensus sequences were inserted in Netprimer software (www.premierbiosoft.

Figs 4-6. Fig. 4: *Cichla kelberi* Kullander & Ferreira, 2006; Fig. 5: *Cichla piquiti* Kullander & Ferreira, 2006; Fig. 6: *carijó*.

Tab. I. Methodologies conditions (PCR, PCR-multiplex and PCR-RFLP).

PCR AND PCR-MULTIPLEX (24µL)				
Concentration conditions		Thermalcycle conditions		
1xPCR buffer	1x	5min	95°C	1 cycle
MgCl2	1.5mM	30s	95°C	35 cycles
dNTP	150µM	30s	52°C	
primers	0.2µM	30s	72°C	
Taq polymerase	0,5U	5min	72°C	1 cycle
DNA template	20-50ng			
RFLP (8µl)				
Concentration conditions		Thermalcycle conditions		
Enzyme buffer (10U/µL)	1x		NlaIV	BsrI
Enzyme (NlaIV/BsrI)	5U	60min	37˚C	65˚C
PCR product	60ng	20min	65˚C	80˚C

Tab. II. Primers utilized in this study.

Primers	Type	Sequence	References
COI-Fish2 F	Universal	5'TCGACTAATCATAAAGATATCGGCAC3'	Ward *et al*., 2005
COI-Fish2 R		5'ACTTCAGGGTGACCGAAGAATCAGAA3'	Ward *et al*., 2005
RAG1*Cichla* F*		5'GGCTCTCTGGATGGTCTTCCT3'	Present study
RAG1*Cichla* R*		5'ACACTTCYCCAATYTCATCCTGGA3'	Present study
COI Ck F	Species-specific	5'ATGATCGGAGGCTTTGGAAAC3'	Present study
COI Cp F		5'GGTGTGTCCTCAATCCTGGT3'	Present study
RAG1 Ck F		5'AACCCCTTTTCTGAGTCCGTA3'	Present study
RAG1 Cp F		5'TACATCTGCACTCTATGTGACTCA3'	Present study

com/netprimer) and species-specific primers (COI Ck F and RAG1 Ck F for *C. kelberi* and COI Cp F and RAG1 Cp F for *C. piquiti*) were designed internally to the universal primers, to produce different sizes fragments for each species (Fig. 7, Tab. II). Posteriorly, consensus sequences were inserted in the Nebcutter V2.0 software (Vincze *et al*., 2003) and restriction maps were generated, thus enzymes with different cleavage patterns for each species were selected (Fig. 8).

PCR-multiplex (Tab. I) and PCR-RFLP (Tab. I) reaction conditions were standardized (performed successfully in every sample). Intraspecific variability was not detected for the analyzed loci, demonstrating that diagnostic nucleotides for each species were fixed over all the studied samples. DNA fragment sizes were determined by electrophoresis on agarose gel stained with SYBR® Safe DNA gel stain, visualized with UV illumination and images were captured by a digital camera (C-5060 5.1 Megapixel, Camedia, Olympus).

RESULTS

Results obtained from genetic analyses revealed the presence of mutational sites that differentiate *Cichla kelberi*

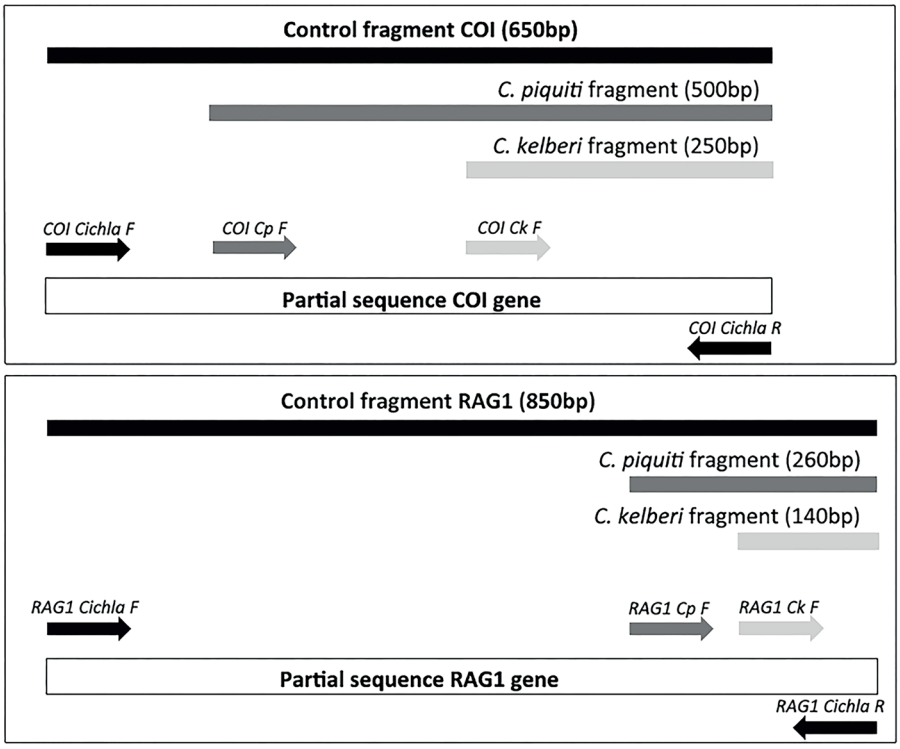


Fig. 7. Recognition and orientation sites of universal and species-specific primers within regions of the mitochondrial (*COI*) and nuclear (*RAG1*) genes of *Cichla* Bloch & Schneider, 1801 species.

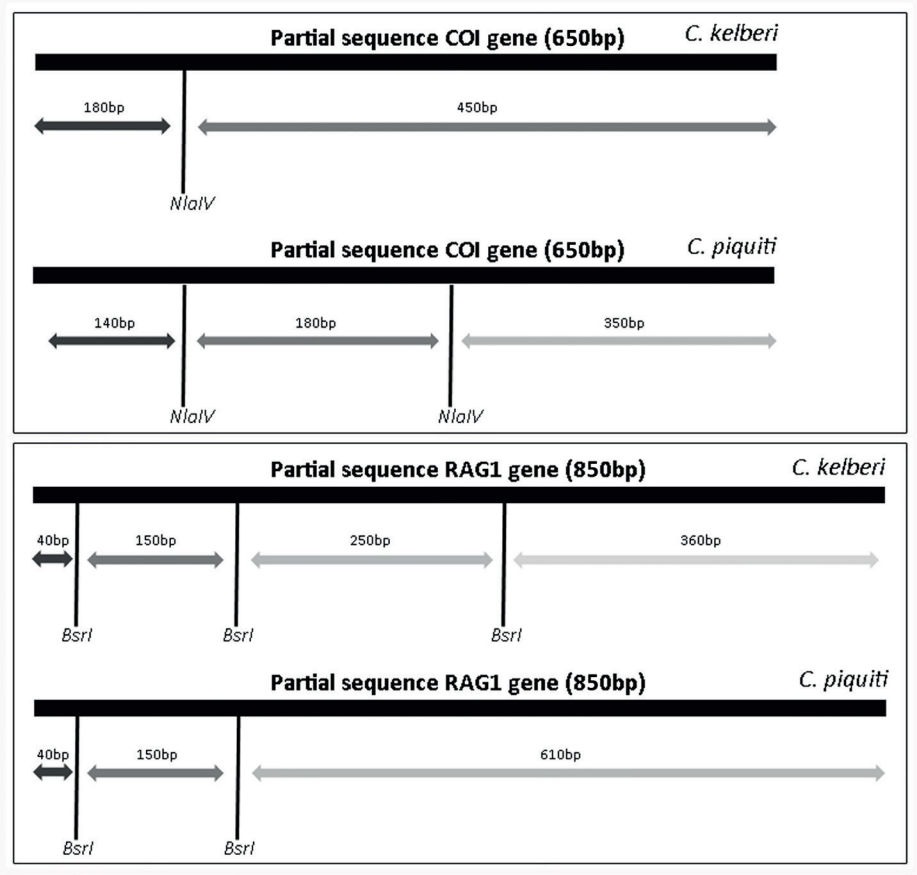


Fig. 8. Restriction maps of the *COI* gene and *RAG1* gene for the species *Cichla kelberi* Kullander & Ferreira, 2006 and *Cichla piquiti* Kullander & Ferreira, 2006.

from *Cichla piquiti* in all the analyzed samples, allowing the development of specific PCR-multiplex and PCR-RFLP markers of COI and RAG1 genes for species identification (Figs 7, 8; Tabs I, II).

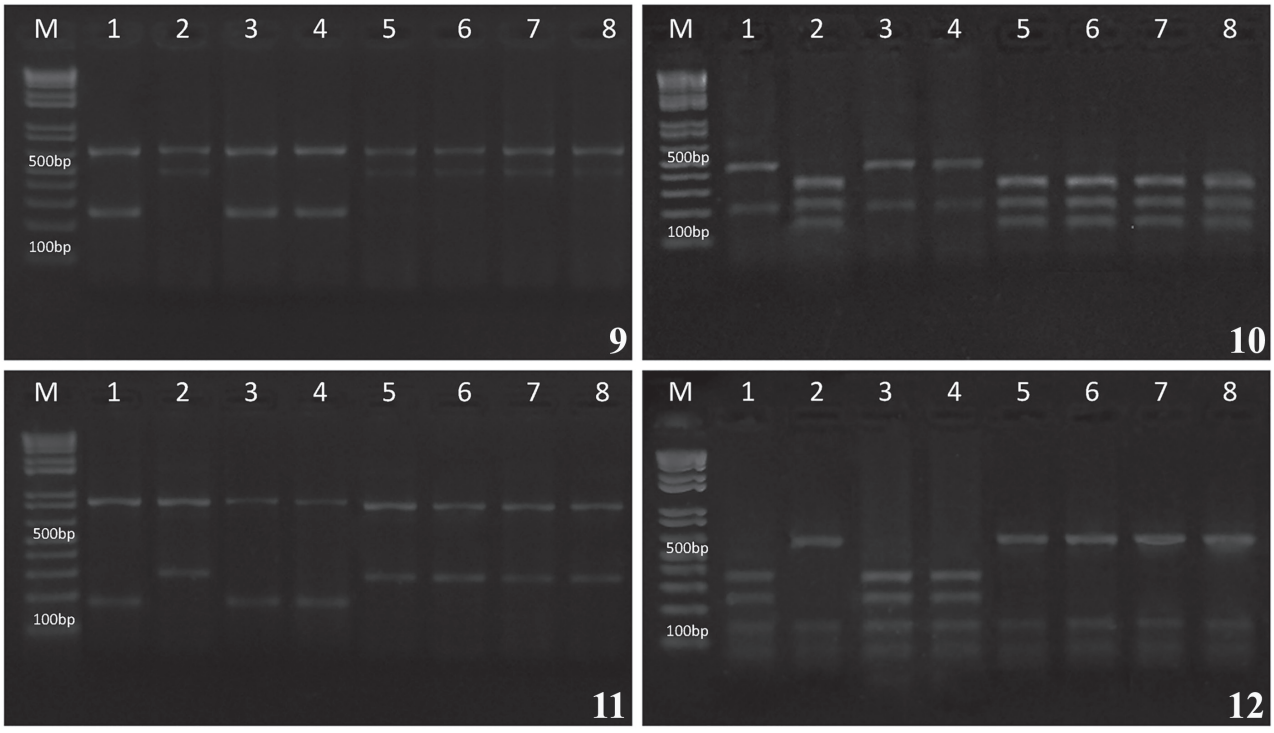
Genetic analysis using both the PCR-multiplex and PCR-RFLP of mitochondrial and nuclear markers clearly differentiated *Cichla kelberi* from *Cichla piquiti* while the possible hybrids identified as *carijó* showed identical genetic patterns to *C. piquiti* in all the analyses (Figs 9-12). Considering details of each molecular technique, the PCR-multiplex of the COI gene (mitochondrial gene) revealed one control band of about 650bp in all samples and a band of almost 250bp for *C. kelberi* and approximately 500bp for *C. piquiti* (Fig. 9). The PCR-multiplex of the RAG1 gene (nuclear gene) revealed one control band of about 850bp in all samples and a band of almost 140bp for *C. kelberi* and approximately 260bp for *C. piquiti* (Fig. 10). The *carijó* individuals also presented identical bands as found in *C. piquiti* for both markers (Figs 9, 10).

The PCR-RFLP of the COI gene (mitochondrial gene) revealed fragments of almost 180 and 450bp for *C. kelberi*, while *C. piquiti* showed bands of approximately 140, 180 and 350bp (Fig. 11). The PCR-RFLP of the RAG1 gene (nuclear gene) revealed fragments of almost 40, 150, 250 and 360bp in *C. kelberi*, while for *C. piquiti* fragments were of approximately 40, 150 and 610bp (Fig. 12). The *carijó* individuals presented identical bands as found in *C. piquiti* for both nuclear and mitochondrial markers when using PCR-RFLP technique (Figs 11, 12).

The utilization of morphometric and meristic data proposed by Kullander & Ferreira (2006) allowed the correct identification of the species *C. kelberi*, *C. piquiti* and the *carijó* individuals. Results of PCA revealed the existence of morphological differences between the analyzed individuals. The first PCA axis highlights morphological difference of *C. kelberi* in relation to *C. piquiti* and *carijó*, with the higher explanation percentage (50%). The second PCA axis shows the morphological differences between *C. piquiti* and *carijó*, with a lower explanation percentage (13%) (Fig. 13). Cluster similarity of the meristic data generated two major groups: a cluster composed mostly by *C. kelberi* individuals and another cluster formed entirely by the two morphotypes of *C. piquiti* (Fig. 14). Even though these characters are used as diagnostic in determining species of the genus *Cichla*, the existence of overlapping prevents the separation of *C. piquiti* and *carijó*.

DISCUSSION

The intermediate morphology to the *C. piquiti* and *C. kelberi* species presented by *carijó* individuals and the hybridization reported in previous studies for *Cichla* (e.g. Alves & Feldberg, 1998; Brinn *et al.*, 2004; Oliveira *et al.*, 2006, 2008; Almeida-Ferreira *et al.*, 2011) made us consider the hybridization hypothesis in this study. However, all the performed analyses do not confirm *carijó* individuals as an interspecific hybrid between *C. piquiti* and *C. kelberi*. Therefore, the findings do not support the hypothesis of



Figs 9-12. Fig. 9, patterns of PCR-multiplex for the *COI* mitochondrial gene. Fig. 10, PCR-RFLP patterns of the *COI* gene with NlaIV enzymes. Fig. 11, patterns of PCR-multiplex for the *RAG* nuclear gene. Fig. 12, PCR-RFLP patterns of the *RAG* gene with the BsrI enzyme. The species are indicated as: column 1, *Cichla kelberi* Kullander & Ferreira, 2006; column 2, *Cichla piquiti* Kullander & Ferreira, 2006; column 3 and 4, *Cichla kelberi*; column 5 and 6, *Cichla piquiti*; column 7 and 8, *carijó*; M, 1 kb molecular weight marker.

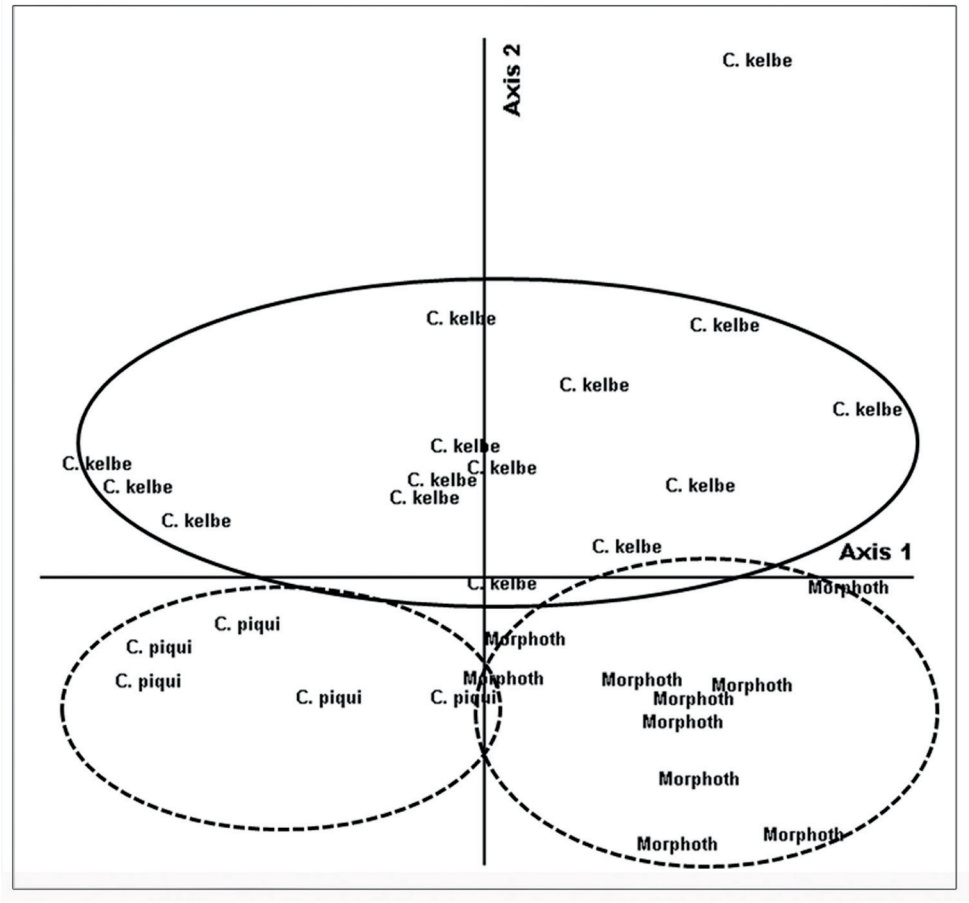


Fig. 13. Principal Component Analysis (PCA) for *Cichla* Bloch & Schneider, 1801 specimens using morphological data. Highlight: *Cichla kelberi* Kullander & Ferreira, 2006, *Cichla piquiti* Kullander & Ferreira, 2006 and *carijó* samples distribution.

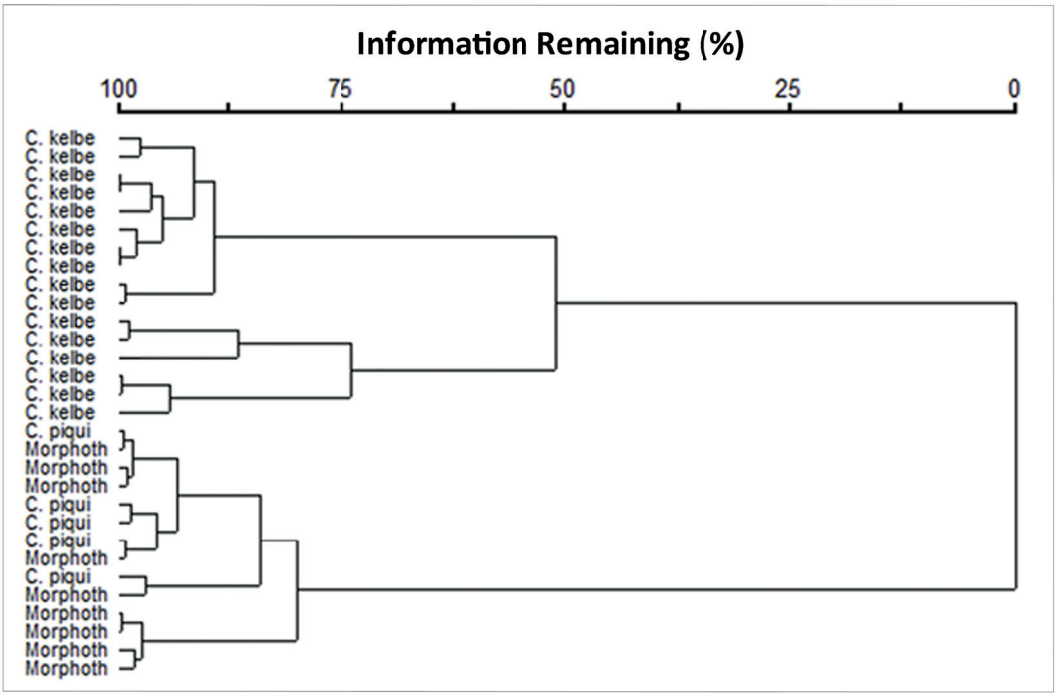


Fig.14. Cluster similarity based on the meristic data of *Cichla* Bloch & Schneider, 1801 specimens, indicating the relationship among *Cichla kelberi* Kullander & Ferreira, 2006, *Cichla piquiti* Kullander & Ferreira, 2006 and *carijó* samples.

hybridization occurring between the two studied species.

The genetic analyses performed in this study clearly differentiated *C. kelberi* from *C. piquiti*, but showed *carijó* as belonging to the *C. piquiti* species, because the electrophoretic profile of *carijó* was identical to that of *C. piquiti*. Besides that, the morphologic analysis revealed two groups, the *C. kelberi* cluster and the *C. piquiti* cluster. The *C. kelberi* cluster formed entirely by *C. kelberi* individuals and the *C. piquiti* cluster formed by two morphotypes, the *C. piquiti* itself and the *carijó*. Thus, both results support the *carijó* as morphotype of *C. piquiti*.

The morphotype confirmation has great biological value for the group because a hybridization confirmation has been a difficult and common problem for the conservation of fishes (Allendorf *et al*., 2001). Considering the high prolificity and the lentic environments adaptations of *Cichla* (Godinho *et al*., 1994), hybridization could be even greater problem. However, studies that consider hybridizations among other species of the genus are of great value, because there are many cases already reported for the group (e.g. Alves & Feldberg, 1998; Brinn *et al*., 2004; Oliveira *et al*., 2006; Oliveira *et al*., 2008; Almeida-Ferreira *et al*., 2011).

In general, this phenotypic changes occurred in morphotypes may be associated to agonistic behavior, spawning, sexual selection (Barlow, 2000) or to sexual dimorphism (Winemiller, 2001; Kullander & Ferreira, 2006). According to Kullander & Ferreira (2006) the juvenile and adult phases of *Cichla* have different colourations. Additionally, Winemiller (2001) describes distinct colourations in adults related to the different stages of gonadal maturation. In addition, the phenotypic changes may be associated to cannibalism inhibition (Zaret, 1977), predatory pressure (Magurran, 2005), predator-prey interactions (Maan & Sefc, 2013) or, yet, to seasonal maturity in both sexes (Reiss *et al*., 2012). In any case, the morphotypes presence is an event already observed in the genus *Cichla* (Winemiller, 2001; Reiss *et al*., 2012).

The present study does not question the reason for the *Cichla* morphotype appearing; it just describes and reports its existence in addition to test whether it constitutes a hybrid species. Studies that promote the understanding of the ecological and reproductive role of this morphotype and how the biotics and abiotics factors influence the appearing of morphotypes such as the *carijó* individuals are of broad interest. For example, different experiments investigating the hypothesis associated with the causes of morphotypes occurrence may include exposing *C. kelberi, C. piquiti* and *carijó* to: agonistic and spawning behavior, absence or presence of sexual selection, seasonal maturity, stage of maturation and reproduction and predatory pressure and interactions, among others.

The new molecular markers propose in the present study might be interesting taxonomically because is the first molecular identification tool developed for *carijó* individuals and the first document relating and analyzing the *carijó*. Therefore, these markers could be used for *Cichla* identification in future research, monitoring, management and conservation programs, because there are scarce studies assigned to the Neotropical cichlids biology, especially involving *peacock bass*, that has great socio-economic value.

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