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Prospecting molecular markers to distinguish *Cichla kelberi*, *C. monoculus* and *C. piquiti*

Luciano Seraphim Gasques[1*], Thomaz Mansini Carrenho Fabrin[2], Daniela Dib Gonçalves[3], Sônia Maria Alves Pinto Prioli[4] and Alberto José Prioli[4]

*[1]Universidade Paranaense, Praça Mascarenhas de Moraes, 4282, 87502-210, Umuarama, Paraná, Brazil. [2]Programa de Pós-graduação em Ecologia de Ambientes Aquáticos Continentais, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. [3]Programa de Pós-graduação em Ciência Animal, Universidade Paranaense, Umuarama, Paraná, Brazil. [4]Programa de Pós-graduação em Biologia Comparada, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. *Author for correspondence. E-mail: lsgasques@yahoo.com.br*

ABSTRACT. Peacock bass, a fish of the genus *Cichla*, is an exotic species from the upper river Paraná floodplain in which the species *Cichla kelberi* and *C. piquiti* have been confirmed, coupled to the specie *C. monoculus* upstream in the Capivara and Taquaruçu dams. The introduction of this genus has caused negative impacts on the diversity of native species. Current research prospects DNA sequences capable of distinguishing the three species and provide molecular data for the taxonomic characterization of the species in the upper Paraná River basin. Sequencing of nuclear (tmo4c4, dlx2 and bmp4) and mitochondrial (cox1, cytb) loci were done from fish of the three species of the genus *Cichla* reported in the literature of the upper Paraná River basin. Sequence analysis provided molecular differentiation for the species through the usage of *loci* cytb, dlx2 and cox1. Since the latter only distinguished *C. piquiti* from the other *Cichla* species, the *loci* bmp4 and tmo4c4 were not adequate to accomplish our aim.

Keywords: mitochondrial and nuclear DNA, exotic species, peacock bass, cytb, dlx2, cox1.

Prospecção de marcadores moleculares para distinção de *Cichla kelberi*, *Cichla monoculus* e *Cichla piquiti*

RESUMO. O tucunaré é um peixe pertencente ao gênero *Cichla*, sendo exótico na planície de inundação do alto rio Paraná, onde foi confirmada a presença das espécies *Cichla kelberi* e *C. piquiti* e, logo a montante, *C. monoculus*, nas represas de Capivara e Taquaruçu. A introdução deste gênero tem ocasionado impacto negativo à diversidade de espécies nativas. Visando providenciar dados moleculares para auxiliar na caracterização taxonômica das espécies deste gênero na bacia do alto rio Paraná, os objetivos deste trabalho foram prospectar sequências de DNA capazes de distinguir as três espécies. Procedeu-se o sequenciamento de *loci* nucleares (tmo4c4, dlx2 e bmp4) e mitocondriais (cox1, cytb) de peixes das três espécies do gênero *Cichla* relatadas na literatura da bacia do alto rio Paraná. As análises das sequências possibilitaram a diferenciação molecular para estas espécies pela utilização dos *loci* cytb, dlx2 e cox1; este último, somente para distinguir *C. piquiti* das demais espécies de *Cichla*. A utilização dos *loci* bmp4 e tmo4c4 não foi adequada para este propósito.

Palavras-chave: DNA mitocondrial e nuclear, espécie exótica, tucunaré, cytb, dlx2, cox1.

Introduction

Fish of the genus *Cichla* belong to the order Perciformes and the family Cichlidae, popularly known in Brazil as peacock bass (Tucunaré). The family is one of the main models of speciation in evolutionary biology due to its diversification on the Great Lakes of Africa, which led to the formation of species with different ecological adaptations in a relatively short period of geological time (JOYCE et al., 2011). Thus, it has been focused upon by many phylogenetic researches (LÓPEZ-FERNÁNDEZ et al., 2010).

The genus *Cichla*, widely distributed in neotropical regions, derives from a monophyletic lineage and is considered the baseline genus for a divergence of neotropical cichlids (FARIAS et al., 2001). The genus has 15 described species which are native to Tocantins, Amazon and Orinoco river basins (KULLANDER; FERREIRA, 2006). Since genetic analysis has shown genetic introgression among some of the species, the validity of this classification has been questioned (WILLIS et al., 2012).

The introduction of species in the upper Paraná River basin, developed by hydroelectric companies

(ESPÍNOLA et al., 2010), is a strategy to increase fish stocks which started in the 1990s (AGOSTINHO et al., 2007). However, the origin of this genus in the Paraná river basin is officially unknown, except for some reports of accidental tanks leaks involving *Cichla monoculus* (Block & Schneider, 1801) during the January 1997 flood in the Paranapanema and Tibagi rivers (ORSI; AGOSTINHO, 1999).

Other relevant aspects related to the illegal introduction of the species are related to its characteristics as sports fishing, due to its aggressiveness when caught with artificial baits, and to the commercial acceptance of its meat (WINEMILLER, 2001, AGOSTINHO et al., 2007; PELICICE; AGOSTINHO, 2009).

The genus *Cichla* is documented in the Upper Paraná River floodplain since the 1990s with special reference to *Cichla kelberi* and *C. piquiti* (KULLANDER; FERREIRA, 2006; GRAÇA; PAVANELLI, 2007). Moreover, a third species, *C. monoculus*, has been documented in the Paraná River upstream segment (BRIÑEZ et al., 2013).

The capture of a great number of juveniles of the genus in the above-mentioned environment has shown a high degree of the species´ reproduction success in the Upper Paraná River floodplain (ESPÍNOLA et al., 2010). The hybridization between *C. kelberi* and *C. piquiti* recorded by genetic researches is another important event that has occurred in the floodplain (ALMEIDA-FERREIRA et al., 2011; OLIVEIRA et al., 2008, 2006).

Combination analyses of molecular markers that identify regions which display different evolutionary rates are necessary to understand the complex evolutionary process in neotropical fish (FABRIN et al., 2014; BEHEREGARAY, 2008) and its hybridization dynamics (MIMS et al., 2010; STREELMAN et al., 2004). In fact, genetic studies are important to provide data that may be used in taxonomic characterization, supporting ecological studies aiming at the management of hybrid populations.

Current research prospects DNA sequences capable of distinguishing the three species *C. kelberi*, *C. piquiti* and *C. monoculus*.

Material and methods

Samples

Tissues preserved in alcohol 70% from the tissue bank of the Genetics Laboratory of The Research Center of Limnology Ichthyology and Aquaculture (NUPELIA), State University of Maringá, were used for DNA extraction. *Cichla monoculus* specimens from the Paranapanema River (n = 7) and Taquaruçu reservoir (Upper Parana River basin), used by Briñez et al. (2013), were retrieved, coupled to *Cichla kelberi* (n = 6) and *C. piquiti* (n = 6) from Tocantins-Araguaia basin, used by Oliveira et al. (2006). Figure 1 demonstrates the sites from where the specimens were collected.

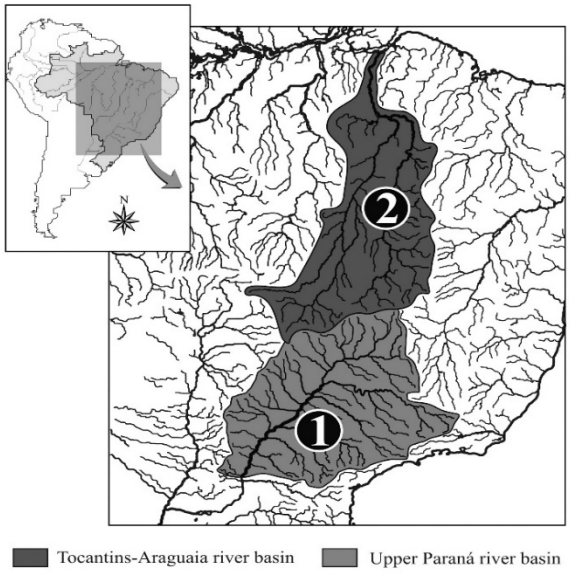

Figure 1. Collection sites of *Cichla* specimens. 1 = *C. monoculus* was retrieved from the river Paranapanema (22°54'S; 52°00'W); 2 = *C. kelberi* and *C. piquiti* were retrieved from the Tocantins-Araguaia basin (09°45´S; 48°22´W).

DNA amplification and sequencing

DNA extraction followed method by Prioli et al. (2002) and the Polymerase Chain Reactions (PCRs) were done in a total volume of 25 µL containing Tris-KCl (20 mM Tris-HCl pH 8.4 with 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 µM of each primer, 0.1 mM of each dNTP, 2.5 U of Taq DNA polymerase, 15 ng of DNA and Milli-Q water to complete the volume.

The nuclear *locus* tmo4c4 was amplified according to Schelly et al. (2006); primers described by Streelman et al. (1998) were used for *locus* tmo4c4; and primers described by Smith et al. (2008) were used for *loci* bmp4 and dlx2. The mitochondrial cytochrome oxidase I sequences (cox1) were amplified as described by Steinke et al. (2009) and the cytochrome b (cytb) region as described by Kocher et al. (1989).

Temperature conditions and time used in the amplification of the respective regions were appropriate and specified in Table 1. The amplified genes were sequenced individually in both directions using the primers already described (Table 2) with Big Dye Terminator kit. The nucleotide sequence determination was conducted in MegaBace automated sequencer (Amersham), according to the manufacturer's instructions.

Table 1. PCR reaction conditions for the different regions of mitochondrial and nuclear *Cichla* DNA.

Amplified region	Initial denaturation		PCR cicles conditions						Cycles	Final ext.	
			Denaturation		Annealing		Extension				
	Temp.	t	Temp.	t	Temp.	t	Temp.	t		Temp.	t
tmo4c4	95ºC	300 s	94 ºC	15 s	48 ºC	5 s	72 ºC	30 s	40	72 ºC	300 s
bmp4	95 ºC	60 s	95 ºC	30 s	55 ºC	60 s	72 ºC	90 s	35	72 ºC	300 s
dlx2	95 ºC	60 s	95 ºC	30 s	55 ºC	60 s	72 ºC	90 s	35	72 ºC	300 s
cox1	94 ºC	120 s	94 ºC	30 s	52 ºC	40 s	72 ºC	60 s	35	72 ºC	300 s
cytb	94 ºC	120 s	94 ºC	30 s	52 ºC	40 s	72 ºC	60 s	35	72 ºC	300 s

Temp. is given in Celsius degrees and t indicates the time in seconds.

Table 2. Primers used in the amplifications for the different regions of mitochondrial and nuclear *Cichla* DNA.

Region	Primer	Sequence (5´-3´)
tmo4c4	tmo4c4 f1-5	CCTCCGGCCTTCCTAAAACCTCTC
	tmo4c4 r1-3	CATCGTGCTCCTGGGTGACAAAGT
bmp4	bmp4 2fb	AACCTCACCAGCATTCCAGA
	bmp4 2r	ATCGCTGAAGTCCACGTAC
dlx2	dlx2 f760	GAAGAGAGYGAGCCAGAAATC
	dlx2 r2	AGTTTGCCAAAAACGACGACGAA
cytb	H15149	CCCCTCAGAATGATATTTGTCCTCA
	L14841	CCATCCAACATCTCAGCATGATGAAA
coxI	H7152	CACCTCAGGGTGTCCGAARAAYCARAA
	L6448-F2	TCGACTAATCATAAAGATATCGGCAC

Sequences analysis

Sequences were edited with BioEdit software while alignment (Clustal W) and distance *p* were determined with MEGA 6 software (TAMURA et al., 2013). Distance matrices were provided in the supplementary material.

Alignments were previously evaluated by the best-fit model test in MEGA 6 software to elaborate the phylogenetic reconstructions. Each was conducted through its bases substitution model by the maximum likelihood method. Each phylogeny used as a parameter 1000 bootstrap re-samplings with the presence of an outgroup (preferably of the same family and with closer phylogenetic analysis, considering the GenBank availability) and also conducted in MEGA 6. The phylogenetic analysis elucidated the clades, whilst bootstraps rates determined the validity of clades distinction. Table 3 provides the GenBank accession number of the sequences obtained in current study.

Table 3. GenBank accession number of specimens sequenced in current study.

Spp.	GenBank accession number of the sequences				
	coxI	cytb	dlx2	bmp4	tmo4c4
Cm1	KT382886	KT382901	KT382948	KT382915	KT382934
Cm2	KT382887	KT382902	KT382949	KT382916	KT382935
Cm3		KT382903	KT382950	KT382917	KT382936
Cm4	KT382888	KT382904	KT382951	KT382918	
Cm5		KT382905	KT382952	KT382919	KT382937
Cm6	KT382889	KT382906	KT382953	KT382920	
Cm7	KT382890		KT382954	KT382921	
Ck8	KT382891	KT382907	KT382955	KT382922	KT382938
Ck9	KT382892	KT382908		KT382923	KT382939
Ck10		KT382909	KT382956	KT382924	KT382940
Ck11	KT382893	KT382910	KT382957	KT382925	KT382941
Ck12	KT382894			KT382926	KT382942
Ck13		KT382911	KT382958	KT382927	KT382943
Cp14	KT382895	KT382912	KT382959	KT382928	KT382944
Cp15	KT382896	KT382913	KT382960	KT382929	
Cp16	KT382897	KT382914	KT382961	KT382930	KT382945
Cp17	KT382898		KT382962	KT382931	KT382946
Cp18	KT382899		KT382963	KT382932	KT382947
Cp19	KT382900		KT382964	KT382933	

Results

The regions cox1 and cytb were individually evaluated for alignment. The number of variable sites was checked and evaluated according to the best evolutionary model. Sequences obtained from the region cox1 (544 bp) revealed 57 variable sites (10.47%). The Hasegawa-Kishino-Yano model with gamma distribution for the reconstruction of the mitochondrial phylogeny of the three species of *Cichla* from cox1 region was employed. Figure 2 provided the results of the phylogenetic reconstruction by the maximum likelihood method.

The second prospected mitochondrial region was related to cytochrome b (cytb) (464 bp) gene. Due to the low number of sequences obtained from the surveyed specimens, three sequences from GenBank (gi294471571, gi294471583 and gi294471565), one from each species, were included for comparison. Figure 2 shows the phylogenetic reconstruction of this region and was based on Hasegawa-Kishino-Yano model, performed by the maximum likelihood algorithm with 1000 bootstrap re-samplings.

Further, tmo4c4, dlx2 and bmp4 were the evaluated nuclear genome regions and the best evolution model, the number of variable sites and

size of alignment were calculated for each region. After calculating the best evolution model for each region, the phylogenies were reconstructed with the maximum likelihood method, taking into consideration 1000 bootstrap re-samplings. Figure 3 demonstrates results for each region.

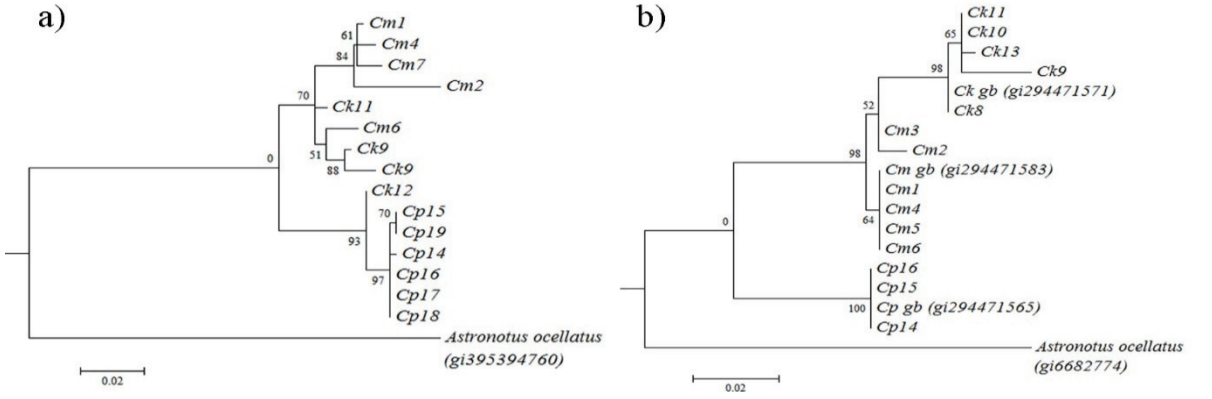

Figure 2. a) Phylogenetic reconstruction of the cox1 (544 bp) gene from specimens of the species *Cichla monoculus* (Cm), *C. kelberi* (Ck) and *C. piquiti* (Cp) by the maximum likelihood method (1000 bootstrap re-samplings) with the Hasegawa-Kishino-Yano model with gamma distribution. *Astronotus ocellatus* was used as outgroup. b) Phylogenetic reconstruction of cytb (464 bp) gene from specimens of the species *C. monoculus* (Cm), *C. kelberi* (Ck) and *C. piquiti* (Cp) by the maximum likelihood method (1000 re-samplings of bootstrap) with the Hasegawa-Kishino-Yano model. *Astronotus ocellatus* was the outgroup.

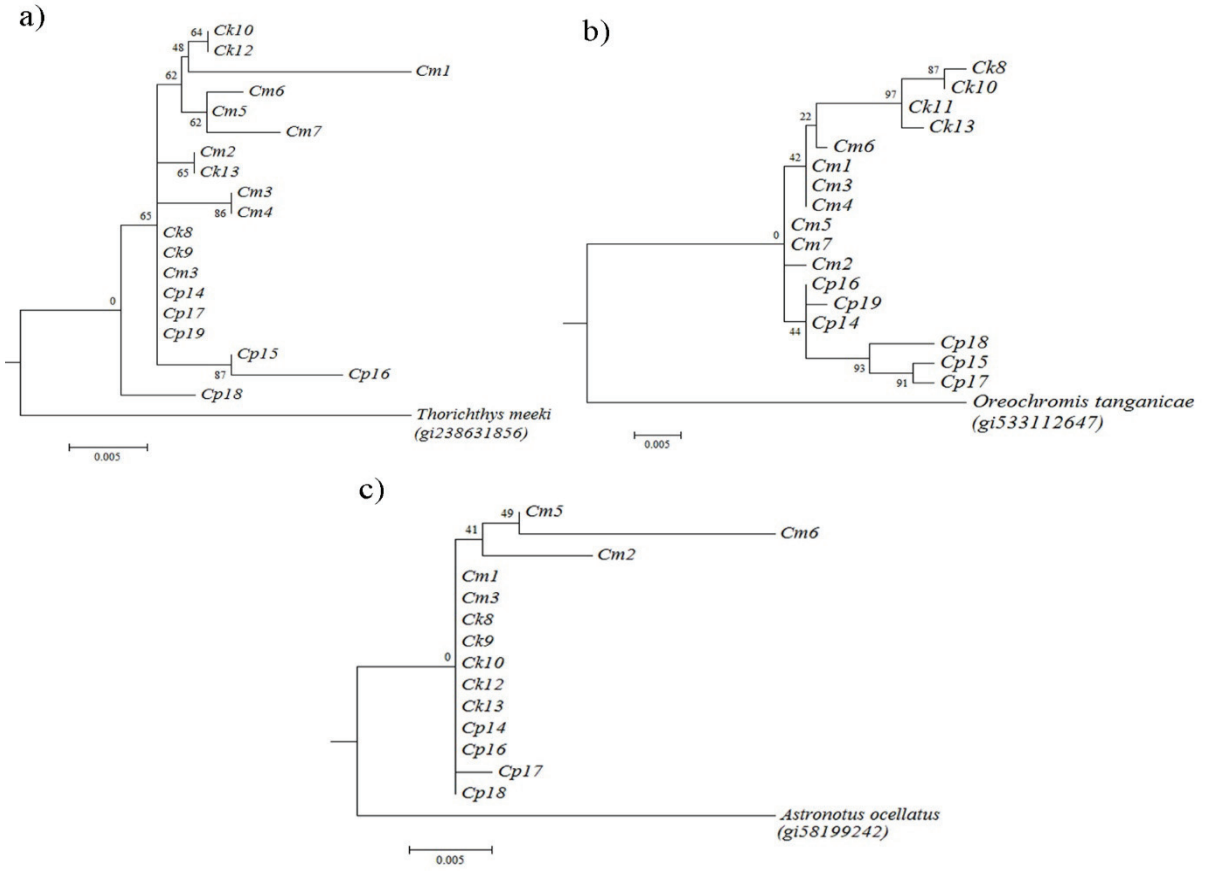

Figure 3. a) Phylogenetic reconstruction of tmo4c4 (523 bp) gene from specimens of the species *Cichla monoculus* (Cm), *C. kelberi* (Ck) and *C. piquiti* (Cp) by the maximum likelihood method (1000 bootstrap re-samplings) with the Juke-Cantor model. *Astronotus ocellatus* was used as outgroup. b) Phylogenetic reconstruction of dlx2 (499 bp) gene from specimens of the species *C. monoculus* (Cm), *C. kelberi* (Ck) and *C. piquiti* (Cp) by the maximum likelihood method (1000 bootstrap re-samplings) using the Kimura-2-parameter model. *Oreochromis tanganicae* was used as outgroup. c) Phylogenetic reconstruction of the bmp4 (441 bp) gene from specimens of species *Cichla monoculus* (Cm), *C. kelberi* (Ck) and *C. piquiti* (Cp) by the maximum likelihood method (1000 bootstrap re-samplings) with the Juke-Cantor model. *Thorichthys meeki* was used as outgroup.

Among the smaller interspecific variation with less than 1%, the *loci* tmo4c4 and bmp4 were highlighted for all evaluated species. The accumulated variations at these *loci* do not appear to be enough for a sharp distinction since rates are close to the intraspecific variation ones.

Although the region with the highest intraspecific variation was cox1 (3.9%) for *C. kelberi*, it was less than or equal to interspecific rates discovered in this region for the combination with the other two species, perhaps due to the fact that the sequence of bases of specimen Ck12 differed a lot when compared to other specimens. The above may be observed in this group by the high standard deviation of the intrapopulational distance p (2.5%).

The lower intraspecific variations occurred in the species *C. piquiti*, in the cytb and tmo4c4 sequences (0 and 0.1%, respectively); tmo4c4 (0.1%) had the lowest rates for *C. kelberi*; whereas the greatest intraspecific distance rates (all greater than 4%) were from cox1 and cytb.

When interspecific and intraspecific average rates from the different prospected regions were compared, three *loci* showed lower internal species variations than the rate reported among the groups. Consequently, the three species could be differentiated. Although the mentioned regions were cytb, cox1 and dlx2, only cytb presented a significant difference among the maximum distance p values. cytb had the interspecific variation at least 2.15 times higher than the intraspecific variation rates. Differences between the rates of the maximum distance *p* were lower for regions cox1 and dlx2, with 1.07 and 1.125 times respectively.

However, other *loci* may be useful to distinguish the species and to evaluate the distances p between pairs of species. Species *C. monoculus* and *C. piquiti* could be segregated in the region cox1. The variation between the intra- and inter-specific distances *p* from these species is at least 2.12 times. However, it is still not possible to clearly distinguish *C. kelberi* from the others in this region since the intra- and inter-specific variations are very close to each other.

Among the nuclear *loci* evaluated, dlx2 seems to be the most promising when it comes to species differentiation. The variation of the groups´ internal distance p was 0.7% for *C. monoculus*, 0.5% for *C. kelberi* and 1.6% for *C. piquiti.* When the genetic distances among the groups were compared, it could be perceived that they were at least 1.12 times different, if the smallest change that occurred in *C. monoculus* was considered with the other species. Table 4 shows the interspecific polymorphic sites between *C. monoculus*, *C. kelberi* and *C. piquiti*.

In the case of the nuclear region tmo4c4, low intraspecific variation is extant for *C. piquiti*, with low interspecific variation rates, and thus low variability in this region. The above indicates that the *locus* may not be suitable for the differentiation of these species. The evaluation of region bmp4 points in the same direction as the tmo4c4 analysis. Although the distance p variation for the region bmp4 indicates a distinction between *C. piquiti* and *C. kelberi,* a more precise evaluation shows the overlap of specimens of these species, which invalidates the use of this marker in the distinction.

Discussion

Although the cox1 region has been widely used to identify species and has even been used as the base sequence in the 'Barcodes of Life Data System Project' (BOLD) (RATNASINGHAM; HERBERT, 2003), the three species *C. kelberi*, *C. piquiti* and *C. monoculus* could not be identified by this region because the sequences failed to have enough variance to differentiate *Cichla monoculus* from *Cichla kelberi*. A similar result occurred for the cox1 region in the case of another cichlid genus (*Oreochromis*), in which the divergence was not significant to differentiate all the evaluated species (WU; YANG, 2012). Nevertheless, the region analysis could distinguish *Cichla piquiti* from the others.

The phylogeny based on the cytochrome b gene region (cytb) indicated the possibility of distinguishing specimens of the three species. Further, the spreading of specimens showed well-defined groupings for the three species. Since there is a clear divergence of species, the region´s polymorphism seems to characterize them clearly.

The analysis of cytb region has been used in phylogenetic studies since it contains low and high

Table 4. Interspecific polymorphic sites of the cytb, cox1 and dlx2 sequences.

	cytb																						cox1		dlx2			
Species	1 0	1 6	2 4	5 2	6 1	8 2	9 4	1 0 3	1 0 6	1 3 3	1 0 0	1 9	1 1	1 0	2 3	2 4	2 5	2 5	2 6	2 8	2 9	3 1	1 5 6	4 8 9	3 8	2 0 2	2 1 2	2 1 7
Cichla monoculus	T	A	G	A	A	A	G	T	G	G	T	A	A	A	T	G	A	G	G	A	A	G	A	C	A	A	G	R
Cichla kelberi	T	.	.	G	.	.	.	.	.	.	.	G	.	.	.	A	.	.	T	.	.	A	G	G	T	G	T	C
Cichla piquiti	A	G	A	.	G	G	A	C	T	A	C	G	G	G	C	.	T	T	.	T	G	.	G	G	G	.	.	R

variable regions, that is, more preserved regions and regions of wider domain. It may be thus considered a good marker for the study of molecular phylogeny in monophyletic families (FARIAS et al., 1999, 2001; GENNER et al., 2007; MUSILOVÁ et al., 2008; PUEBLA, 2009; SMITH et al., 2008). This region has also been used in biogeographical studies of the *Cichla* genus and demonstrated its potential for this purpose (WILLIS et al., 2007).

Although there is the distinction for the three species with *locus* dlx2, the region would be safer to distinguish between *C. piquiti* and *C. kelberi.* The gene is part of the group of 'dlx homeobox' gene family and is involved in the embryonic formation of the brain, jaws and teeth of vertebrates (PANGANIBAN; RUBESTEIN, 2002). In Lake Malawi´s cichlids, the analysis of these sequences was able to distinguish two species with introgressive hybridization (MIMS et al., 2010). Further, it has been used for cichlids phylogeny in other research works (HULSEY et al., 2010; SMITH et al., 2008).

The nuclear locus tmo4c4 showed random and very low variability, preventing the species distinction process. The locus is known because of its low similarity to TINTIN proteins becoming an immunoglobulin domain. The region does not seem to be associated with positive selection (STREELMAN et al., 1998); for closely related species, the intraspecific diversity may be equal to interspecific differences.

The impossibility of 'bone morphogenic protein 4' (bmp4) gene usage may be due to its morphological differences among different species. The gene has been effective when used as a parameter for the study of the genetic relationships in other fish groups, such as the genus *Scarus* (SMITH et al., 2008). When East African cichlids were evaluated, the bmp4 gene could be found mainly associated with species that have undergone a rapid speciation and a high replacement rate (PUEBLA, 2009). This feature does not appear to be associated with the case of the genus *Cichla*, perhaps because the morphological characteristics of this group are not as diverse as African cichlids.

Phylogeny results show a greater proximity between *C. kelberi* and *C. monoculus*, which may be evidenced by the phylogenies presented to these *loci*. Results corroborate those reported by Willis et al. (2010) who, using the mitochondrial DNA controlling region, posited similarly the two species within the same cladze, whereas they placed *C. Piquiti* in another one.

When the mitochondrial regions were evaluated, although the positioning of the species evaluated for mitochondrial cox1 region was the same discovered for nuclear *loci*, it was possible to perceive an interesting difference in assessing the phylogeny of cytb *locus*. A greater proximity between *C. monoculus* and *C. piquiti* than *C. monoculus* and *C. kelberi* could be observed. The fact that there are discrepancies between the reconstructed phylogenies based on nuclear *loci* and mitochondrial is common (TOEWS; BRESFORD, 2012). However, in current study, the mitochondrial *locus* cox1 points at the same nuclear direction, while cytb points towards a discrepant one. Species closeness is another aspect that should be underscored.

Conclusion

Since the effectiveness of different *loci* in differentiating *Cichla monoculus*, *C. kelberi* and *C. piquiti* species is tested, the analysis foregrounds distinction between *C. monoculus* and *C. kelberi* from *C. piquiti* by cytb and dlx2 *loci* sequences, and the difference between *C. piquiti* from the other two species of the genus *Cichla* present in the upper Paraná River basin by cox1.However, data rejected the use of bmp4 and tmo4c4 *loci* to identify these species.

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