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DNA barcode of Parodontidae species from the La Plata river basin - applying new data to clarify taxonomic problems

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In the past years, DNA barcoding has emerged as a quick, accurate and efficient tool to identify species. Considering the difficulty in identifying some Parodontidae species from the La Plata basin and the absence of molecular data for the group, we aimed to test the effectiveness of DNA barcoding and discuss the importance of using different approaches to solve taxonomic problems. Eight species were analyzed with partial sequences of Cytochrome c oxidase I. The mean intraspecific K2P genetic distance was 0.04% compared to 4.2% for mean interspecific K2P genetic distance. The analyses of distance showed two pairs of species with K2P genetic divergence lower than 2%, but enough to separate these species. *Apareiodon* sp. and *A. ibitiensis*, considered as the same species by some authors, showed 4.2% genetic divergence, reinforcing their are different species. Samples of *A. affinis* from the Uruguay and Paraguay rivers presented 0.3% genetic divergence, indicating a close relationship between them. However, these samples diverged 6.1% from the samples of the upper Paraná River, indicating that the latter represents a potentially new species. The results showed the effectiveness of the DNA barcoding method in identifying the analyzed species, which, together with the morphological and cytogenetic available data, help species identification.

Nos últimos anos o DNA barcoding surgiu como uma ferramenta rápida, precisa e eficiente para identificar espécies. Considerando a dificuldade na identificação de algumas espécies de Parodontidae da bacia do rio da Prata e da ausência de dados moleculares para o grupo, testamos a eficácia do código de barras de DNA e discutimos a importância do uso de diferentes abordagens para resolver problemas taxonômicos. Oito espécies foram analisadas com sequencias parciais do gene citocromo c oxidase I. A distância genética média K2P intraespecífica foi de 0,04% comparado com 4,2% para distância genética média K2P interespecífica. As análises de distância mostraram dois pares de espécies com divergência genética K2P inferior a 2%, mas o suficiente para separar estas espécies. *Apareiodon* sp. e *A. ibitiensis*, consideradas a mesma espécie por alguns autores, mostraram 4,2% de divergência genética, confirmando serem espécies diferentes. Amostras de *A. affinis* dos rios Uruguai e Paraguai apresentaram 0,3% de divergência genética, indicando um maior grau de relação entre elas, no entanto, esses exemplares divergiram em 6,1% em relação aos exemplares do alto rio Paraná, o que indica que estes últimos representam uma espécie potencialmente nova. Os resultados mostraram a eficácia do método de DNA barcoding na identificação das espécies analisadas, os quais, em conjunto com os dados morfológicos e citogenéticos disponíveis auxiliam na identificação inequívoca das espécies.

Key words: *Apareiodon*, COI gene, Cytogenetics, Fish identification, Morphology.

Introduction

During the past two centuries, about 1.7 million species were described by taxonomists, but it is known that this number is still quite reduced considering the biological diversity present on Earth (Blaxter, 2003; Wilson, 2003). Although biological research depends greatly on species diagnosis, traditionally performed by taxonomists (most based on analyses of morphological characters), other tools have been created to assist in this difficult task (Hebert *et al*., 2003).

Given the lack of specialists in many groups and geographic areas and the insufficient funds for taxonomic studies (Godfray, 2002; Mallet & Willmott, 2003; Pires & Marinoni, 2010), the use of integrated methodologies is increasingly necessary and a great call for the deployment of "Integrative Taxonomy" is actually stronger. According to Dayrad (2005), this new approach involves multiple and complementary perspectives (phylogeography, comparative morphology, genetics, ecology, development, behavior, among others) and has successfully assisted traditional

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morphology in studies on biodiversity (Baker *et al*., 2003; Marcussen, 2003; Meyer, 2003; Malhotra & Thorpe, 2004; Gibbs, 2009; Jansen *et al*., 2009; Chen *et al*., 2011). Bickford *et al*. (2007) conveyed that in the past two decades there has been an exponential increase in the identification and recognition of cryptic species with the advancement and use of molecular tools. Examples of such integration are increasingly common in fish, such as in the description of *Gymnotus sylvius* (Albert *et al*., 1999), *Gymnotus pantanal* (Fernandes *et al*., 2005), *Moenkhausia forestii* (Benine *et al*., 2009), and *Tetragonopterus carvalhoi* (Melo *et al*., 2011). These examples are particularly relevant, since they refer to new species from quite complex fish genera which were described after the accumulation of morphological and molecular evidence, demonstrating the singularity of the studied samples regarding their respective counterparts.

Among the molecular methodologies used for species identification, the DNA barcoding emerges as a quick, accurate and efficient tool, and, as such, contributes greatly to taxonomic studies and biodiversity researches (Hebert & Gregory, 2005; Hajibabaei *et al*., 2007). The basic principle that sustains the DNA barcoding methodology is related to the premise that a short standardized DNA sequence - Cytochrome *c* Oxidase I (COI), can distinguish species through the interspecific genetic variation, which exceeds intraspecific variation (Hajibabaei *et al*., 2007; Toffoli *et al*., 2008). Although the use of DNA barcoding is very controversial for using a single mitochondrial gene for species identification, it is believed to be a powerful tool (Ward *et al*., 2009). Many studies have demonstrated its effectiveness in identifying species in different animal groups (Folmer *et al*., 1994; Hebert *et al*., 2004; Barrett & Hebert, 2005; Hajibabaei *et al*., 2006), including fish (Ward *et al*., 2005; Hubert *et al*., 2008 Persis *et al*., 2009; Pereira *et al*., 2010).

Parodontidae is a group of small-sized fish distributed primarily throughout South America, occurring in the eastern half of Panamá, on the Pacific and Caribbean coasts of Colombia and the Pacific coast of Ecuador, in the Orinoco and Amazonas basins, in the Guianas and southwards, including the La Plata basin (Roberts, 1974). It is a relatively small family that includes three genera: *Parodon*, *Apareiodon*, and *Saccodon* (Pavanelli, 2003). Cytogenetic studies on Parodontidae reveal that although the species of this family show a predominant diploid number of 54 chromosomes, the karyotypic macrostructure and genomic structure are very diversified, with distinct sex chromosome systems (Moreira-Filho *et al*., 1980; Jesus & Moreira-Filho, 2000a; Vicari *et al*., 2006; Rosa *et al*., 2006; Bellafronte *et al*., 2009; 2011), structural polymorphism (Jorge & Moreira-Filho, 2000, 2004), interspecific and intraspecific variation in the number and position of nucleolus organizer regions on chromosomes (Moreira-Filho *et al*., 1984; Vicente *et al*., 2001; Bellafronte *et al*., 2009; 2011) and interspecific differences in the distribution of distinct satellite DNA families (Vicente *et al*., 2003; Bellafronte *et al*., 2011; Schemberger *et al*., 2011).

When comparing results from different techniques (morphological characterization and cytogenetics), it is observed that some species in the Parodontidae family are particularly difficult to identify. Thus, considering this difficulty, the absence of molecular data for the group and given the importance and complexity of the fish fauna of the La Plata River basin, the authors aimed to test the effectiveness of DNA barcoding to identify Parodontidae species present in the La Plata River basin and discuss the importance of using "integrative taxonomy", *i.e*., different approaches on the resolution of taxonomic problems.

Materials and Methods

Species collection. Specimens of seven known and a putative new species of Parodontidae were collected in eight distinct localities along the La Plata basin, including the Uruguay, Paraguay, and Paraná River basins (Table 1, Fig. 1). Fresh tissue samples were taken and preserved in -20ºC absolute ethanol. Vouchers specimens were deposited in taxonomic collections (Table 1) and identified with the assistance of taxonomists.

Extraction, PCR amplification and DNA sequencing. Genomic DNA extraction was performed from solid tissues (liver, muscle or fin) using the phenol: chloroform extraction technique (Sambrook *et al*., 1989). Partial sequences of the cytochrome c oxidase I gene (COI) were amplified with the Fish F1 and Fish R1 primers (Ward *et al*., 2005). The PCR reaction contained the final volume of 25,0 µl, including 2,5µl 10x PCR Buffer, 0,7µl $MgCl_2$ (50mM), 0,5 µl of each primer (10mM), 4,0 µl dNTPs mix (1,25mM), 0,2 µl Taq polymerase (Invitrogen), 1,0 µl template DNA (50ng/ µl) and 15,6 µl ultrapure water. Amplifications were made using the cycle that corresponds to an initial step of 2 min at 94ºC, followed by 35 cycles of 0,5 min at 94ºC, 0,5 min at 54ºC and 1 min at 72ºC, followed by 10 min at 72ºC. PCR products were visualized on 1% agarose gel and purified by the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare Life Sciences). Sequencing reactions of both DNA strands were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit and sequencing was performed on the automatic sequencer ABI 3730 DNA Analyzer (Applied Biosystems).

Data Analysis. Individual sequences of each species were initially analyzed using Bioedit 5.0.9 software (Hall, 1999) and a consensus sequence was obtained for each DNA segment for each species. All the sequences were aligned with the ClustalW algorithm integrated in the software DAMBE (Xia & Xie, 2001). Genetic distances were calculated using the distance model Kimura-2-parameter (K2P) (Kimura, 1980). The Neighbor-joining (NJ) dendrogram with the genetic K2P distances was made to provide a representative graphic of the divergence pattern and separation among species (Saitou & Nei, 1987). The genetic distance analyses and the dendrogram were made with the software MEGA 5.01 (Tamura *et al*., 2011). For the pairs of species that presented interspecific divergence values lower than 2%, the nucleotide diagnostic (ND) approach was applied. The NDs were manually

Table 1. List of specimens used in barcoding analyses and their collection data.

Species	Localities	Coordinates	Vouchers
A. affinis	Cuiabá river , Paraguay basin (MT)	15º37'52.07"S 56º04'55.14"W	Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brasil (NUP 12151)
A. affinis	Arroyo Chimiray river, Uruguay basin (Argentina)	28º05'29.8"S 55º42'19.49"W	Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brasil (NUP 12154)
A. affinis	Passa-Cinco river, Upper Paraná basin (SP)	22º 25'26"S 47º41'56"W	Museu Nacional do Rio de Janeiro (MNRJ: 31428)
A. ibitiensis	Passa-Cinco river, Upper Paraná basin (SP)	22º 25'26"S 47º41'56"W	Museu Nacional do Rio de Janeiro, Brasil (MNRJ32771)
A. piracicabae	Passa-Cinco river, Upper Paraná basin (SP)	22º25'26"S 47º41'56"W	Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brasil (NUP 12149)
A. vitattus	Jordão river, Iguaçu basin (PR)	25°42'31"S 51°53'53"W	Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brasil (NUP 1950)
A. vladii	Piquiri river, Upper Paraná basin (PR)	25°01'40.9"S 52° 27'32.8"W	Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brasil (NUP 3375)
Apareiodon sp.	Verde river, Upper Paraná basin (PR)	25º04'35.59"S 50º04'02.10""W	Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brasil (NUP 3443)
P. moreirai	Sapucaí river, Upper Paraná basin (SP)	22º40'33.76"S 45º41'00"W	Museu Nacional do Rio de Janeiro, Brasil (MNRJ 22557)
P. nasus	Cuiabá river, Paraguay basin (MT)	15°34'40.69"S 56°09'58.73"W	Museu Nacional do Rio de Janeiro, Brasil (MNRJ29787)

identified with the software Bioedit 5.0.9 (Hall, 1999). All sequences obtained in this study were deposited in Barcode of Life Data Systems (BOLD).

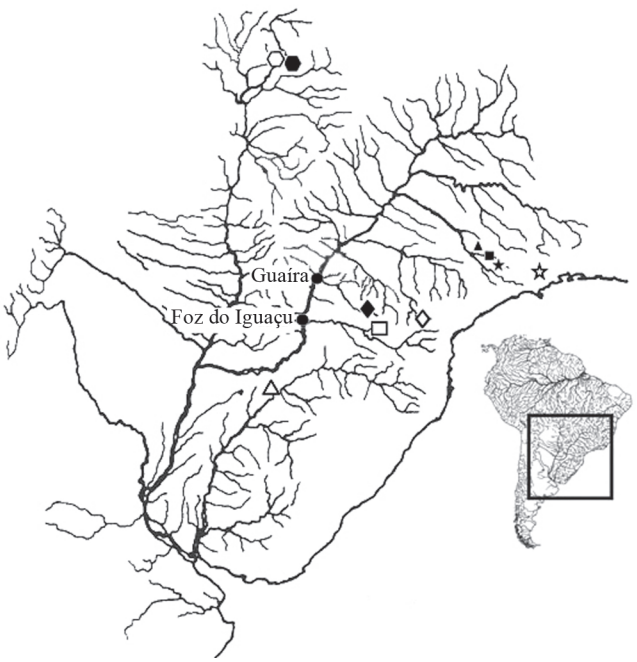

Fig. 1. Map of the La Plata River basin showing the localities of Parodontidae samples used in this study. Empty pentagon: *P. nasus*; full pentagon: *A. affinis*; empty star: *P. moreirai*; full star: *A. affinis*; empty square: *A. vittatus*; full square: *A. piracicabae*; empty lozenge: *Apareiodon* sp.; full lozenge: *A. vladii*; empty triangle: *A. affinis*; full triangle: *A. ibitiensis*. The Iguaçu Falls and the Itaipu hydroelectric Power Plant are located in the city of Foz do Iguaçu, PR and the old Seven Falls in the city of Guaíra, PR - shown on the map.

Results

All species analyzed were represented by more than one specimen (an average of 4.4 specimens per species), totalizing 44 COI sequences with 659 bp. The COI sequences obtained were of high quality and the consensus sequence showed no evidence of insertions, deletions or stop codons. All species were discriminated by their barcode sequences. A Neighbor-Joining tree is presented in Fig. 2. The mean K2P genetic distance within species was 0.04%, comparing to 4.2% mean interspecific K2P genetic distance (Table 2). The distances between congeners ranged from 0.9% to 10.7% among the species of *Apareiodon* and were of only 0.7% between the two analyzed species of *Parodon* (Table 2). Thus, the interspecific variation was 105 times higher on average compared to the intraspecific variation. The genetic distance between the two analyzed genera was 14.1%. Distance analyses showed two pairs of species with K2P genetic divergence lower than 2%, but enough to separate these species (Table 2, Fig. 2). For these pairs of species, the ND approach was applied, showing the presence of five nucleotides for the pair of species *A. piracicabae* and *A. vittatus*, and four for the pair of species *P. morerai* and *P. nasus* (Table 3). The species *A. affinis* showed intraspecific K2P genetic divergence values higher than 2% (Table 2), splitting into three subgroups, according to the NJ dendrogram obtained, corresponding to their collection points (Fig. 2). The analysis of these subgroups conducted separately showed that the subgroup referring to the upper Paraná River population diverges considerably from the other two (6.1%), thus, representing a possible new species (Table 2, Fig. 2).

A table for comparison of different characters used for species identification (chromosomal, molecular, and morphological) was constructed to integrate data of

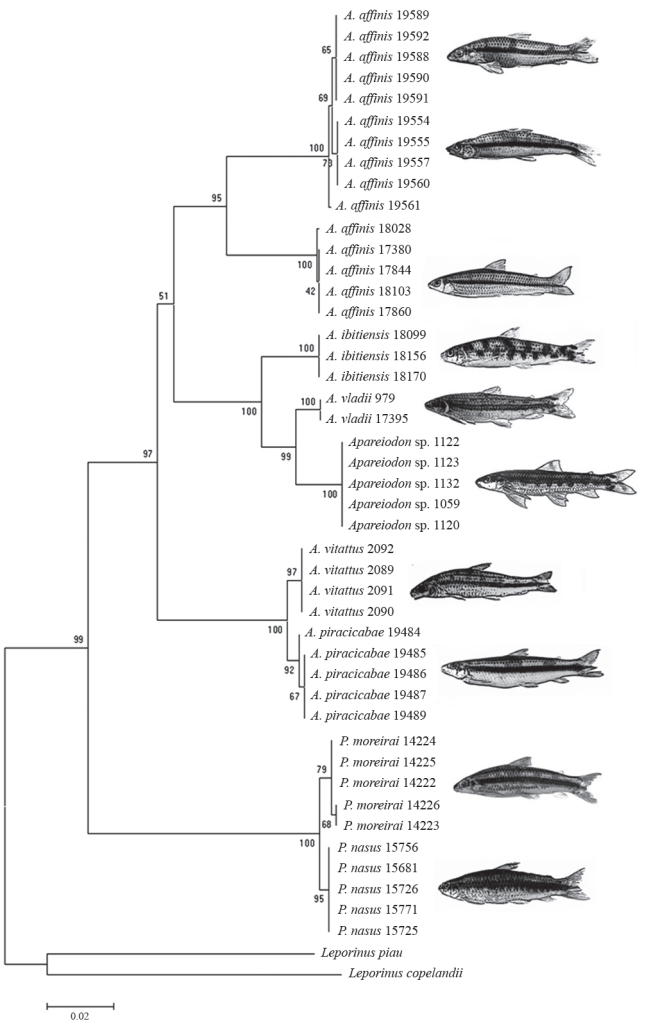

Fig. 2. K2P distance NJ dendrogram showing the nine analyzed species/populations of Parodontidae from the La Plata River basin.

Parodontidae from the literature and the results obtained here (Table 4). The pair of the species *A. ibitiensis* + *Apareiodon* sp. and *A. piracicabae* + *A. vittatus* are chromosomally and morphologically very similar, however the first pair showed a high genetic divergence (4.2%) and the second pair showed a low genetic divergence (0.9%). As for the pair of species the *P. nasus* + *P. moreirai*, although they were very distinct chromosomally and morphologically they showed low genetic divergence (0.7%). The samples of *A. affinis* from the Paraguay and Uruguay rivers, morphologically identical to the upper Paraná River population, showed very distinct chromosomal characteristics and high genetic divergence (6.1%) (Table 4).

Discussion

For the family studied, the DNA barcoding methodology was very efficient, allowing the correct identification of the seven known and a putative new species of Parodontidae. The mean intraspecific K2P genetic divergence was 0.04%, 105 times smaller than the interspecific divergence, which was 4.2%. These values are consistent with other DNA barcoding studies performed on fish. Ward *et al*. (2005) discriminated Australian marine fish species that were analyzed with a mean K2P distance of 0.39% in individuals of the same species compared to 9.93% in species within the genus, thus showing 25 times more variation among congeneric species than among conspecific individuals. Hubert *et al*. (2008), studying continental fish from Canada, discriminated 93% of the species with an average of 0.3% conspecific distance and 8.3% congeneric distance, *i.e*., a difference of 27 times between these two categories. Ward *et al*. (2009) analyzed the K2P genetic divergence values in 1088 fish species available on BOLD and found conspecific values of 0.3% and congeneric values of 8.4%, discriminating 97.5% of the species. Marine fish species from India showed an average K2P distance of 0.24% within species and a

Table 2. Nucleotide divergence among species: The highlighted numbers represent K2P differences lower than 2% between species.

	A. affinis (Uruguay)	*A. affinis* (Paraguay)	*A. affinis* (Upper Paraná)	*A. ibitiensis*	*A. piracicabae*	*Apareiodon* sp.	*A. vitattus*	*A. vladii*	*P. moreirai*	*P. nasus*
A. affinis (Uruguay)	0.001									
A. affinis (Paraguay)	**0.003**	0.000								
A. affinis (Upper Paraná)	0.061	0.061	0.001							
A. ibitiensis	0.091	0.092	0.080	0.000						
A. piracicabae	0.097	0.097	0.085	0.092	0.001					
Apareiodon sp.	0.102	0.103	0.093	0.042	0.107	0.000				
A. vitattus	0.097	0.098	0.084	0.096	**0.009**	0.107	0.000			
A. vladii	0.099	0.099	0.086	0.033	0.096	0.022	0.096	0.000		
P. moreirai	0.153	0.153	0.156	0.146	0.137	0.143	0.133	0.136	0.001	
P. nasus	0.146	0.146	0.153	0.147	0.140	0.142	0.136	0.137	**0.007**	0.000

Table 3. Analysis by nucleotide diagnostic (ND) for the pair of species with divergence lower less than 2%.

	Nucleotide Diagnostic (ND)				
	ND 083	ND 103	ND 226	ND 331	ND 361
A. piracicabae	T	G	G	A	C
A. vittatus	C	A	A	G	T
	ND 178	ND 274	ND 445	ND 628	
P. nasus	G	A	T	T	
P. moreirai	A	G	C	G	

congeneric average of 17.2%, showing a variation of 71X between the conspecific and congeneric distances (Persis *et al*., 2009). Pereira *et al*. (2010) analyzed species from the Paraíba do Sul River basin and found a difference of 79x between the conspecific (0.13%) and congeneric species (10.36%).

The intraspecific divergence value found in the present study is lower than the values found in the literature for groups of fish, while the value found for congeneric species is consistent with other studies. According to Pereira *et al*. (2010), this relatively low value may be attributed to the possibility that the analyzed species show a low divergence among conspecific individuals, or to a limited sampling of the genetic variation of the considered species. Thus, even though all species are represented by more than one specimen, it is possible that increasing the number of specimens would lead to an increase in conspecific values.

DNA barcode- based information should not be considered alone, but as an extension of studies in morphology, behavior, geography, and any other attribute of the organism, which can facilitates its identification (DeSalle, 2006). All species analyzed herein with COI (except for the *A. affinis* population from the Uruguay River) and reliably identified have been chromosomically and morphologically characterized, as shown in Table 4. In some cases, cytogenetics and morphology are in agreement in the characterization of species, such as *A. piracicabae*, *A. vladii*, *A. vitattus*, *P. moreirai*, and *P. nasus*. In other cases, cytogenetics is able to distinguish samples from one species considered by taxonomy as a single entity, for example, the *A. affinis* samples. In this case, DNA barcoding was of great importance for the detection of the putative species (Table 4).

Two pair of species showed low interspecific divergence: *A. piracicabae* and *A. vittatus* (0.9%) and *P. moreirai* and *P. nasus* (0.7%). Although low, these values were enough to discriminate them due to the high difference between the conspecific and congeneric K2P genetic divergences (conspecific values lower than 0.1%). Additionally the ND analysis also allowed their discrimination. Interestingly, the pair of the species *A. piracicabae* and *A. vitattus*, besides having a low K2P divergence, showed a close relationship confirmed by a phylogeny based on morphological characters (Garavello, 1977; Pavanelli, 1999) and on their chromosomal characteristics (Moreira-Filho *et al*., 1985; Jesus & Moreira-Filho, 2000a; Bellafronte *et al*., 2011) (Table 4). However, besides the similarities, *A. piracicabae* and *A. vittatus* are considered valid species. This fact may be related to the emergence of the Iguazu Falls (located in city of Foz do Iguaçu, Paraná State, Brazil), where the fish populations of the Iguaçu River were isolated from those of the Paraná River (Fig. 1). The falls are about 70 m high (Maack, 1981), and probably allowed the differentiation. As for *P. nasus* and *P. moreirai*, morphologically and chromosomally distinct, the low K2P divergence (Table 4) may indicate that separation of these species at the molecular level did not yet occur, and this may be explained by incomplete lineage sorting. This may have happened because there was not enough time for the separation of these two lineages after the speciation event, *i.e*., the absence of gene flow must be recent and insufficiency of time for significant mutations to occur, since these species may be found in sympatry and syntopy in the Sapucaí River, type locality of *P. moreirai* (Ingenito & Buckup, 2005).

In *Apareiodon* sp., a species very similar to *A. ibitiensis*, the mean genetic divergence was high enough to differentiate between them (4.2%). Vicari *et al*. (2006) chromosomically characterized *Apareiodon* sp. collected in the Verde River, a tributary of the Tibagi River (Paraná) belonging to the hydrographic system of the upper Paraná River. According to Pavanelli (pers. comm.), it may be a new species of the genus. Cytogenetically, *Apareiodon* sp. also showed great similarity with *A. ibitiensis*, however with some particularities. Regarding the similarities, both have 54 chromosomes, sharing the same ZZ/ZW single sex chromosome system and the location of the 5S rDNA is on the same chromosome pair (pair 9) characterized by the same heterochromatization process (Table 4). Subtle differences are present in the chromosome morphology (*A. ibitiensis* has 50 meta-submetacentric and 6 subtelocentric chromosomes) and the NORs are single in *Apareiodon* sp. and multiple in *A. ibitiensis* (Table 4) (Moreira-Filho *et al*., 1984, 1985; Jesus & Moreira-Filho, 2000a; Vicari *et al*., 2006; Bellafronte *et al*., 2009). Also, in studies with satellite DNAs, Schemberguer *et al*. (2011) considered *Apareiodon* sp. identical to *A. ibitiensis*. Unlike the previous techniques, DNA barcoding was a more effective and definitive methodology for the differentiation of these two species.

In *Apareiodon affinis*, the COI analysis showed a low divergence among populations of the Uruguay and Paraguay basins (0.3%), indicating a greater relationship among them, but also showed a high divergence (6.1%) between these two populations and that from the upper Paraná River. Moreover, morphological and cytogenetical divergences are also found (Table 4). This species was described by Steindachner in 1879 with type locality in the La Plata River, province of Buenos

Table 4. Table comparing the data from different approaches. References: 1 - Garavello (1977); 2 - Moreira - Filho *et al*. (1980); 3 - Moreira - Filho *et al*. (1985); 4 - Jesus *et al*. (1999); 5 - Pavanelli (1999); 6 - Jorge & Moreira - Filho (2000); 7 - Jesus & Moreira - Filho (2000a); 8 - Jesus & Moreira - Filho (2000b); 9 - Centofante *et al*. (2002); 10 - Pavanelli (2003); 11 - Jorge & Moreira - Filho (2004); 12 - Bellafronte *et al*. (2005); 13 - Ingenito & Buckup (2005); 14 - Vicari *et al*. (2006); 15 - Rosa *et al*. (2006); 16 - Bellafronte *et al*. (2009); 17 - Ingenito (2008); 18 - Bellafronte *et al*. (2011); 19 - Schemberguer *et al*. (2011); 20 - present study.

Species	Collection locality (River-State)	Cytogenetic characters						Molecular characters	Morphological characters	References
		2n Male / female	Karyotypic Formula Male	female	Sexual systems	rDNAs 18S and 5S	Satellites DNAs pPh2004 and Wap	Genetic divergence K2P (COI)	Diagnosis	
Apareiodon affinis	Passa-Cinco River (SP)	54/55	50m/sm+4st	51m/sm+4st	ZZ/ZW$_1$ W$_2$	- pair 26 - pair 8	- pairs 2 and 13 - chromosomes Z, W$_1$, W$_2$; and autosomes.	- 6,1% for *A. afinnis* Paraguay and Uruguay; - 8,8% a 9,9% for congeners; - 14,8% at 15,4% for *Parodon*	It differs from *A. ibitiensis*, *Apareiodon* sp., *A. vladii* and *P. nasus* for not having pigments adjacent to the mid-lateral stripe and/or lateral line. It distinguishes from A. *piracicabae* for possessing 29.5 or more preanal scales (*vs.* less than 29); 12 or more cusps on teeth premaxilla (*vs.* 9-12 cusps), straight- edged teeth (*vs.* round- edged).	1,2, 3, 4, 5,6, 10, 11, 17, 18, 19, 20
Apareiodon affinis	Cuiabá River (MT)	54 /54	a = 42m/sm+12 st/a b = 44m/sm+10 st/a	-	absent		-	-6,1% for *A. afinnis* Upper Paraná; - 9,7% a 10,2% for congeners; - 14,6% at 15,6% for *Parodon*		
Apareiodon affinis	Arroyo Chimiray River (Argentina)	-	-	-	-	-	-			
Apareiodon ibitiensis	Passa-Cinco River (SP)	54 /54	50m/sm+4st	50m/sm+4st	ZZ/ZW	- pairs 6, 14, 15, 26 - pair 9	- absent - chromosomes Z, W and autosomes	- 3,3% at 9,6% for congeners; - 14,6% at 14,7% for *Parodon*	It has a particular staining pattern characterized especially by dark vertical or oblique projections, directed upwards and downwards the lateral line and no longitudinal stripes under those projections, making it distinct from *A. affinis*, *A. vladii*, *A. vitattus*, *A. piracicabae*, *P. nasus*, and *P. moreirai.*	1, 3, 5, 7, 10, 16, 17, 19, 20
Apareiodon sp.	Verde River (PR)	54 /54	48m/sm+6st	47m/sm+7st	ZZ/ZW	- pair 25 - pair 9	- absent - chromosomes Z, W and autosomes	- 2,2% at 10,7% for congeners; - 14,2% at 14,3% for *Parodon*	Great resemblance to *A. ibitiensis*. It differs from it by the presence of the reduced or absent inter-hial structure.	14, 15, 19, 20
Apareiodon vladii	Piquiri River (PR)	54 /54	50m/sm+4st	50m/sm+4st	ZZ/ZW	- pair 26 - pairs 9 and 3	- absent - chromosomes Z, W and autosomes	- 2,2% at 9,6% for congeners; - 13,6% at13,7% for *Parodon*	Presence of one tooth in the jaw *vs.* two in *A. ibitiensis*. and *A. piracicabae*. Standard color ranges without vertical and/or dark stripes, extending below the lateral line separates *A. vladii* from *A. ibitiensis*; more pointed premaxillary teeth, larger median cusp with dentary teeth decreasing abruptly size distinguish A. *vladii* from A. *piracicabae*.	15, 17, 19, 20

Table 4. cont. Table comparing the data from different approaches. References: 1 - Garavello (1977); 2 - Moreira - Filho *et al*. (1980); 3 - Moreira - Filho *et al*. (1985); 4 - Jesus *et al*. (1999); 5 - Pavanelli (1999); 6 - Jorge & Moreira - Filho (2000); 7 - Jesus & Moreira - Filho (2000a); 8 - Jesus & Moreira - Filho (2000b); 9 - Centofante *et al*. (2002); 10 - Pavanelli (2003); 11 - Jorge & Moreira - Filho (2004); 12 - Bellafronte *et al*. (2005); 13 - Ingenito & Buckup (2005); 14 - Vicari *et al*. (2006); 15 - Rosa *et al*. (2006); 16 - Bellafronte *et al*. (2009); 17 - Ingenito (2008); 18 - Bellafronte *et al*. (2011); 19 - Schemberguer *et al*. (2011); 20 - present study.

Species	Collection locality (River-State)	Cytogenetic characters						Molecular characters	Morphological characters	References
		2n Male / female	Karyotypic Formula Male	female	Sexual systems	rDNAs 18S and 5S	Satellites DNAs pPh2004 and Wap	Genetic divergence K2P (COI)	Diagnosis	
Apareiodon piracicabae	Passa-Cinco River (SP)	54 /54	52m/sm+2st	52m/sm+2st	absent	- pair 27 - pair 9	- absent terminal and interstitial sites on pair 27	- 0,9% at 10,7% for congeners; - 14,6% at 14,7% for *Parodon;* - ND between *A. piraciacabae* and *A. vittatus* → 83=T; 103=G; 226=G; 331=A; 361=C	Presence of pigments adjacent to the longitudinal band and/or lateral line, especially downward in *A. ibitiensis*. It features premaxillary teeth with cutting edges without pronounced rounded central cusp, differently from *A. affinis* and *P. nasus* (straight edge) and *A. ibitiensis, Apareiodon* sp. and *A. vladii* (rounded edge with more pronounced central cusp).	1, 3, 5, 7, 10, 17, 18, 19, 20
Apareiodon vittatus	Iguaçu River (PR)	54 /54	52m/sm+2st	52m/sm+2st	absent	- pair 27 - pair 9	- absent - only terminal sites	- 0,9% a 10,7% for congeners; - 13,3% a 13,6% for *Parodon;* - ND between *A. vittatus* and *A. piracicabae* → 83=C; 103=A; 226=A; 331=G; 361=T	Presence of 38-40 lateral line scales (*vs.* 39-46 in *A. affinis*). 26-28 have preanal scales (compared to 30-31 and 32-35 in *A. piracicabae* and *A. affinis*). It features 11 teeth cusps in the premaxillary row (*vs.* 13-15 in *A. affinis* and 10 in *A. ibitiensis*); 6-7 inconspicuous dark crossbars on the back (*vs.* four in *A. piracicabae* and 7-8 in *A. affinis*), and three longitudinal bands on the dark metallic brown shade along the body (compared to two bands in *A. piracicabae* and *A. ibitiensis* and one in *A. affinis*).	1, 7, 10, 17, 18, 19, 20
Parodon moreirai	Sapucaí River (SP)	54 /54	54m/sm	54m/sm	ZZ/ZW	- pair 15 - pair 11	- pair 9 and chromosomes Z and W - chromosomes Z, W (syntenic to pPh2004) and autosomes	- 0,7% for congeners; - 13,3% a 15,4% for *Apareiodon;* - ND between *P. morerai* e *P. nasus* → 178=G; 274=A; 445=T; 628=T	Presence of a large and continuous mediolateral band, absent in *P. nasus*. *Parodon moreirai* differs from *A. ibitiensis* by the presence of a dark, medio-lateral line, continuous and well-defined in *A. affinis*, and *A. piracicabae* and by a dark longitudinal stripe down the midlateral band extending beyond the anal fin, occasionally joining the mediolateral band above this fin (*vs.* absence or suavity of this stripe, never exceeding pelvic fins). In *A. vittatus*, the biggest difference is in the interorbital distance, from 36.3 to 40.4% (against 32.2 to 34.5%).	9, 13, 17, 19, 20
Parodon nasus	Cuiabá River (MT)	54 /54	48m/sm+6st	48m/sm+6st	absent	- both of pair 25 (syntenic)	- pairs 6, 13, 26 and 27 - pair 13 (syntenic to pPh2004) and other autosomes	- 0,7% for congeners; - 13,6% a 14,8% para *Apareiodon*; - ND between *P. nasus* and *P. morerai* → 178=A; 274=G; 445=C; 628=C	Presence of premaxillary teeth with a straight cutting edge, distinct from *A. ibitiensis*, *Apareiodon* sp., and *A. vladii* (rounded edge with a more pronounced central cusp) and from *A. piracicabae*, *A. vitattus* and *P. moreirai* (rounded edge without a pronounced central cusp). It differs from *A. affinis* by its vertically elongated lateral spots (*vs.* no lateral spot).	3, 5, 8, 10, 12, 17, 18, 19, 20

Aires (Argentina) and has a wide geographic distribution, being found in several locations along the La Plata River basin, including the Lower Paraná, Uruguay, Paraguay, and upper Paraná basins (Pavanelli, 2003; Buckup *et al*., 2007). Although different populations have not shown morphological differences (Pavanelli, 1999, 2003), chromosomal analyses showed differences among the *A. affinis* samples from the lower Paraná River, Paraguay, and upper Paraná basins. While the samples from the lower Paraná and Paraguay show 54 chromosomes, with the presence of acrocentric chromosomes, the upper Paraná sample is composed of 54/55 chromosomes in males and females, respectively, characterizing a ZZ/ZW_1W_2 multiple sex chromosome system (Table 4). Thus, the high genetic divergence, along with the cytogenetical data, reinforces the probability that the *A. affinis* samples from the upper Paraná are of a distinct group, therefore suggesting a taxonomic revision. Until 1982, before the construction of the Itaipu hydroelectric dam, located in the city of Foz do Iguaçu, Paraná State, Brasil (Fig. 1), the Guaíra Falls, an important geographical barrier, considered the world's largest waterfall by the volume of water, was located in the city of Guaíra, Paraná State (Fig. 1), and acted isolating the majority of the upper Paraná river ichthyofauna from the remaining fauna downstream: lower Paraná/Uruguay/Paraguay basins, including the *A. affinis* populations (Bonetto *et al*., 1986; Abell *et al*., 2008).

Although large migratory fishes were able to cross the Guaíra Falls in both directions, and despite the fact that the damming led to the invasion of essentially sedentary or short-distance migratory species, the upper Paraná River is considered a discrete ichthyological ecoregion (Hubert & Reno, 2006; Júlio Jr *et al*., 2009). Regarding the populations from the Uruguay and Paraguay basins analyzed herein, the low divergence observed probably occurred because of the opportunity of dispersal through the La Plata River, an unaltered route in the past 10 million years (Sivasundar *et al*., 2001).

DNA barcoding, besides discriminating species, is also sometimes able to differentiate higher taxonomic levels such as genera, families, orders and classes, characterized by the constant increase in genetic variation (Hubert *et al*., 2008). The mean between the genera *Parodon* and *Apareiodon* was 14.4%, compatible with a clear separation between them, such as the mean found by Ward *et al*. (2005) for the genera of Australian marine fish (15.46%) and Hubert *et al*. (2008) for the continental freshwater fish from Canada (15.38%). Therefore, the data presented here suggest that *Apareidon* and *Parodon* are distinct units within the family Parodontidae. In this case, chromosomal characters overlap, not being able to sustain a differentiation between the genera.

In summary, the DNA barcode studies provide examples of the usefulness of this methodology to catalog the marine and continental waters fish diversity, since it supports the discovery of probable new species and genera, provides information about species that represent good models for comparative biogeographical studies, and also provides knowledge about the molecular systematics of fish species complexes, contributing to improve the estimates of local species richness, assists in the delineation of taxonomic units for conservation programs and clearly discriminates commercially important fishes (Beheregaray, 2008; Carvalho *et al*., 2011). In this study, the integrated analysis of morphological, cytogenetical and molecular characters (DNA barcode) was important for the characterization of taxonomically problematic species of Parodontidae. Therefore, these identification systems, as well as others in integrative taxonomy, should not be seen as competitors or exclusive, but as different approaches cooperating to reach the same goal - delimiting species (Dayrat, 2005).

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