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DNA barcoding confirms the occurrence of *Rhamdia branneri* and *Rhamdia voulezi* (Siluriformes: Heptapteridae) in the Iguaçu River Basin

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DNA barcoding is a widely utilized molecular-based identification of species and taxonomic resolutions. Until recently, *Rhamdia voulezi* and *Rhamdia branneri* were considered species synonyms of *Rhamdia quelen*; however, morphological and cytogenetic analyses have suggested the validity of distinct species. Due to the absence of molecular taxonomy of *R. voulezi* and *R. branneri*, the objective of this study was to test its validity through traditional DNA barcoding and the GMYC (General Mixed Yule Coalescent) COI-based analyses in 19 specimens from the Iguaçu River Basin. In both methodologies, three MOTUs (Molecular Operational Taxonomic Units) were identified based on the estimated optimum threshold (OT = 0.77). The average inter-MOTU distance (NJ, K2P) between *R. branneri* and *R. voulezi* was 1.4%, and 0% intra-MOTU distance in both species. The two species identified as *R. branneri* and *R. voulezi* showed correspondence with taxonomic and morphological identifications. With regard to *R. quelen*, the average intra-MOTU distance was greater than OT (2.7%), indicating that this species can be formed by different MOTUs. We suggest that molecular and taxonomic studies should be employed concurrently in *R. quelen*, to prevent contamination of wild species by hybridizations.

Keywords: Aquaculture, COI, Jundiá, MOTU, Species complex.

O DNA barcoding é uma ferramenta molecular precisa para a identificação de espécies e resoluções taxonômicas. Até recentemente, *Rhamdia voulezi* e *Rhamdia branneri* eram consideradas sinônimas de *Rhamdia quelen*, contudo caracteres morfológicos e citogenéticos têm apontado à validade de ambas. Devido à escassez de informações sobre a taxonomia molecular de *R. voulezi* e *R. branneri*, o objetivo do presente estudo foi testar a validade das mesmas através do método de DNA barcoding tradicional e GMYC (*General Mixed Yule Coalescent*), por meio da análise do gene COI em 19 espécimes do rio Iguaçu. Em ambos os métodos, três MOTUs (Unidades Taxonômicas Operacionais Moleculares) foram identificadas com base no ótimo *threshold* (OT = 0,77). A média inter-MOTU (NJ, K2P) entre *R. branneri* e *R. voulezi* foi 1,4%, com valores de 0% intra-MOTUs em ambas espécies. As duas espécies identificadas como *R. voulezi* e *R. branneri* apresentaram correspondência com a identificação taxonômica e morfológica dos respectivos *vouchers*. No que se refere a *R. quelen*, os resultados intra-MOTU foram superiores ao OT (2,7%), evidenciando a possibilidade de existirem diferentes MOTUs denominadas como *R. quelen*. Sugerimos que estudos moleculares e taxonômicos sejam empregados em *R. quelen*, para evitar a contaminação de espécies selvagens por hibridizações.

Palavras-chave: Aquicultura, COI, Complexo de espécies, Jundiá, MOTU.

Introduction

DNA barcoding is a widely molecular-based identification system utilized to identify biological specimens based on a short standardized sequence of DNA (Hebert *et al.* 2003a,b). The integration between the molecular and traditional taxonomic identification is a relevant association and can promote the identification of a greater number of species than currently described (Steinke, Hanner, 2011). Presently, there is a range of studies addressing the Neotropical ichthyofauna through DNA barcoding, suggesting and revealing several new species (Pereira *et al.*, 2011; Rosso *et al.,* 2012; Gomes *et al.,* 2015; Ramirez, Galetti Jr., 2015; Melo *et al.*, 2016). However, the success of the identification of species from molecular data depends on the precise taxonomic identification and availability of reference sequences (Ward *et al.*, 2009).

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The genus *Rhamdia* was considered the most specious in the Heptapteridae family, with approximately 100 species, in which the vast majority of species has great similarities in body shape, color pattern and habitat use. In a review carried out by Silfvergrip (1996), all described species were narrowed down to only 11 valid species, in which 49 species were synonimized as *Rhamdia quelen*. Among the clustered species discussed by Silfvergrip (1996), *Rhamdia branneri* Haseman, 1911 and *Rhamdia voulezi* Haseman, 1911, both from Iguaçu River Basin, were also recognized as synonyms of *R. quelen* (Quoy, Gaimard, 1824). Nevertheless, recent cytogenetic and morphometric studies have disagreed with Silfvergrip's revision. The characteristics associated with chromosome B and karyotype variations between species of the genus *Rhamdia* from the Iguaçu River, for example, showed that *R. voulezi* and *R. branneri* are distinct from *R. quelen* (Abucarma, Martins-Santos, 2001; Garcia *et al.*, 2010). Recently Mise *et al.* (2013) and Garavello, Shibatta (2016) detected evident morphometric characteristics for the reappraisal of *R. branneri* and *R. voulezi* from the Iguaçu River Basin.

According to Garavello, Shibatta (2016), *R. voulezi* and *R. branneri* exhibit some morphometric differences in the length of the adipose fin and the maxillary barbel as well as a deeper caudal peduncle (for more details see Garavello, Shibatta, 2016). According to those authors, both species differ from *R. quelen* by the fine serrate margin of pectoral-fin spine, dorsal dark-brown or light-gray coloration along body, and abdomen pale, without profuse small black spots. Prior to being considered synonyms of *R. quelen*, *R. branneri* and *R. voulezi* were described as endemic to the Iguaçu River Basin, Southern Brazil (Garavello, Shibatta, 2007; Garavello *et al.*, 2012; Baumgartner *et al.*, 2012; Garavello, Shibatta, 2016).

Thus, given the taxonomic evidence supported by recent studies, we used DNA barcoding and species delimitation methods to confirm the validity of *R. branneri* and *R. voulezi*. These results will contribute to the knowledge of this complex genus, which may be formed by a larger number of species, which are currently cultivated and commercialized only as the common name, jundiá.

Materials and methods

Sampling and taxonomic identification. Nineteen specimens of *R. voulezi* and *R. branneri* from the Iguaçu River Basin (Tab. 1) were sampled. Of these, three vouchers were deposited in the ichthyological collection of the Museu de Zoologia of the Universidade Estadual de Londrina (one *R. voulezi* (MZUEL 16503, specimen 3619) and two *R. branneri* (MZUEL 16504, specimens 3630 and 3638)). The specimens were identified following Haseman (1911) and Garavello, Shibatta (2016). Principal Components Analysis was employed to describe morphometric differences among vouchers of *R. branneri* and *R. voulezi*, using the program PAST (Hammer *et al*., 2001) on 11 log transformed morphometric variables.

Tab. 1. Specimen information, voucher identification and BOLD accession numbers for the *Rhamdia branneri* and *Rhamdia voulezi* analyses. All samples were collected by Robie Bombardelli.

Samples	BOLD access	Species	Locality	River	Latitude	Longitude	Voucher Number
3621	BRH001-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3630	BRH002-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3631	BRH003-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3632	BRH004-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3633	BRH005-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3634	BRH006-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3635	BRH007-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3636	BRH008-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3638	BRH009-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3640	BRH010-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3640	BRH011-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3641	BRH012-16	*R. branneri*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16504
3614	BRH013-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503
3616	BRH014-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503
3619	BRH015-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503
3624	BRH016-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503
3625	BRH017-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503
3628	BRH018-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503
3629	BRH019-16	*R. voulezi*	Salto Osório Reservoir	Iguaçu	-25.621003	-52.620797	MZUEL 16503

DNA barcoding analysis. Total genomic DNA was extracted from fin tissues preserved in ethanol using a method described by Aljanabi, Martinez (1997). Partial sequences of the mitochondrial gene COI were amplified using the Fish-F1 and Fish-R1 primers (Ward *et al.*, 2005), following the PCR method proposed by Bellafronte *et al.* (2013). PCR products were visualized on a 1% agarose gel and purified with 20% PEG (Lis, 1980). Sequencing reactions were performed using BigDye TM Terminator v 3.1 (Cycle Sequencing Ready Reaction Kit, Applied Biosystems), and the PCR products were sequenced for both strands in ABI 3500XL (Applied Biosystems). Contigs were then assembled and edited using BioEdit v. 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). BOLD accession numbers are given in Tab. 1.

We combine our dataset with the following sequences available in the public reference database BOLD system (http://www.boldsystems.org): *Rhamdia guatemalensis* (code HBGM) as outgroup, and *Rhamdia quelen* (we used samples from two different basins due to broad distribution of this species: code BSB, Upper Paraná River Basin and code ITAPE, Itapecuru River Basin). The traditional DNA barcoding method was based on the genetic distance between a pair of sequences, using the Kimura 2-parameter (K2P) model. The expectation of this method is that individuals of the same species have lower genetic distance (intraspecific variation) compared to individuals of other species (interspecific variation) (Hebert *et al*., 2003a), based on chosen threshold. The optimum threshold (OT; Collins *et al*., 2012) was calculated using the *localMinima* function, using the R package SPIDER (*Species IDentity and Evolution*; Brown *et al.*, 2012). SPIDER is a R package implementing a number of useful analyses for DNA barcode studies and research into species delimitation and speciation, with functions essential for generating statistics from DNA barcode data, allowing optimizing divergence threshold limits based on the concept of the barcoding gap (Meyer, Paulay, 2005). For details about the software and download of package see Brown *et al.*, 2012 and SIPER web site (http://spider.r-forge.r-project.org/SpiderWebSite/spider.html).The advantage of this method is that it does not require a priori knowledge about the identity of the species and minimizes error (barcoding gaps) (Meyer, Paulay, 2005; Brown *et al.*, 2012). The optimum threshold value was used to define the molecular operational taxonomic units (MOTUs) using JMOTU (Jones *et al.*, 2011). The graphical representation of the MOTUs was performed by neighbor-joining analysis (NJ) using the K2P model, implemented in the software Mega 6.6 (Tamura *et al.*, 2013). The support of clades was tested by the bootstrap method containing 10,000 pseudo replicates.

The General Mixed Yule Coalescent (GMYC) is a phylogenetic approach for species delimitation based on single-locus data that combines a coalescent model that is a relative robust tool for species delimitation (Pons *et al.*, 2006; Fujisawa, Barraclough, 2013). This approach requires an ultrametric tree and predicts that difference in branching rates between the events of inter-specific or intraspecific relations, based on the length of the branches (Pons *et al*., 2006). The ultrametric tree was generated in BEAST v.2.2.1 (Bouckaert *et al.*, 2014), with the substitution model calculated in the JModelTest 2.1.4 (Posada, 2008 - HKY+G), using relaxed molecular clock with a lognormal distribution and birth-death model. Three independent runs were carried out with 10 million generations each. Posteriorly, the runs were combined using the LogCombiner v.1.8.3 (Drummond *et al*., 2012), with a burn-in of 25%, as method suggested by Machado *et al*. (2016). Data mixing and effective sample size (ESS) were verified in Tracer v1.5. GMYC was carried out in SPLITs (Species Limits by Threshold Statistics; Monaghan *et al.*, 2009) with RStudio (http://r-forge.r-project.org/ projects/splits), using the unique threshold method to detect the transition point between intra- and interspecific relationships.

Results

Taxonomy. Morphometric characters of specimens deposited as vouchers of *R. branneri* and *R. voulezi* showed the same morphological pattern indicated by Garavello, Shibatta (2016). The first axis of PCA represents the size, evidencing linear differences among *R. branneri* (vouchers MZUEL 16504, specimen 3630 with 303.3 mm SL (Fig. 1a) larger than specimen 3638 with 251.0 mm SL) (Fig. 2). Both *R. branneri* differ in body shape from *R. voulezi* specimen 3619 (MZUEL 16503, Fig. 1b) in the second axis of PCA, with the larger distance between the dorsal and adipose fins (5.1% and 6.5% of the standard length (SL), in *R. branneri* versus 9.6% of the SL in *R. voulezi*) (Tab. 2).

DNA barcoding. The sequences from 19 specimens were deposited in the BOLD database, specifically in the project entitled Barcoding *Rhamdia* (BRH). Because there are not insertions, deletions, or stop codons, pseudogenes, were not amplified. The sequences of *R. voulezi* and *R. branneri* resulted in 562 bp, while the sequences obtained from the BOLD database ranged from 582 to 652 bp. All individuals of the genus *Rhamdia* showed 109 positions that were parsimony informative, and nine differed between *R. branneri* and *R. voulezi* sequences. The OT value for the dataset analyzed was 0.77%, and three MOTUs (correspondent to *R. branneri, R. voulezi,* and *R. quelen*) were identified using this cut-off value as a barcode threshold in jMOTU. Phylogenetic trees (NJ and Bayesian inference) showed congruent topology and *were* strongly supported (Fig. 3) for *R. branneri, R. voulezi, R. quelen* from the Upper Paraná basin (BSB) and from the Itapecuru River (ITAPE), and *R. guatemalensis*. If these four species are considered equally valid (*R. voulezi*, *R. branneri*, *R. quelen* and *R. guatemalensis*), only *R. quelen* has an intra-MOTU divergence higher than the OT (2.7%), suggesting the presence of cryptic genetic diversity (Tab. 3). On the other hand, as expected by the DNA barcoding method, inter-MOTU values were greater than the OT values in all pairwise comparisons (Tab. 3).

Fig. 1. Lateral view of **a.** *Rhamdia branneri* (BRH009-16; MZUEL 16504) 303.34 mm SL and **b.** *Rhamdia voulezi* (BRH015-16; MZUEL 16503) 300.8 mm SL from the Iguaçu River Basin, Paraná State, Brazil.

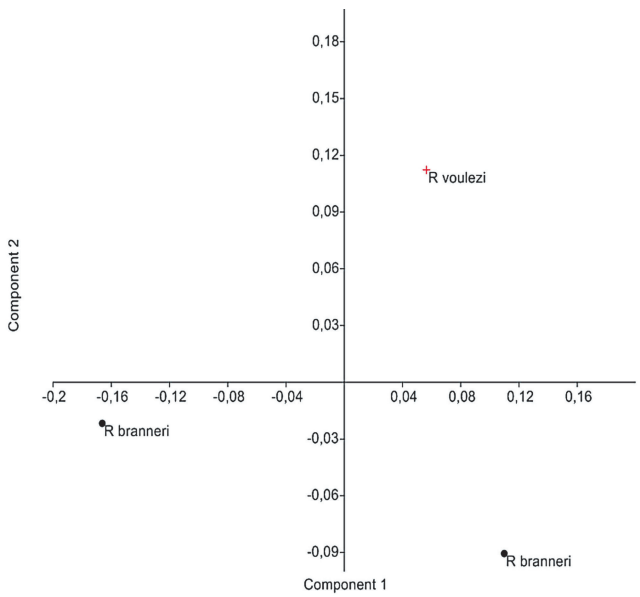

Fig. 2. Principal components analysis of *Rhamdia voulezi* and *Rhamdia branneri* from the Iguaçu River Basin, Paraná State, Brazil.

Tab. 2. Variable loading on the first and second axis of the principal components analysis of *Rhamdia voulezi* and *Rhamdia branneri* from Iguaçu River Basin, Paraná State, Brazil.

Variable	Axis 1	Axis 2
Head length	0.3493	0.0626
Orbital diameter	0.1991	0.2041
Maxillary barbel length	0.2749	-0.3599
Predorsal distance	0.2879	0.0004
Dorsal-fin base length	0.3322	-0.1903
Dorsal fin to adipose fin distance	-0.05536	0.8025
Adipose-fin base length	0.3392	0.1785
Prepelvic length	0.395	-0.0272
Anal-fin base length	0.09651	-0.0556
Caudal peduncle depth	0.304	-0.1189
Caudal peduncle length	0.4401	0.3081
Eigenvalue	0.0214473	0.0106
Percent of variance	66.85%	33.14%

The maximum likelihood for the GMYC model was significantly higher (L= 224.13) than the null model (L_0 = 220.09, p-value = 0.01). The transition point between the coalescence and speciation/extinction processes had close correspondence with the five groups obtained by the traditional DNA barcoding analysis (dotted vertical line in Fig. 3).

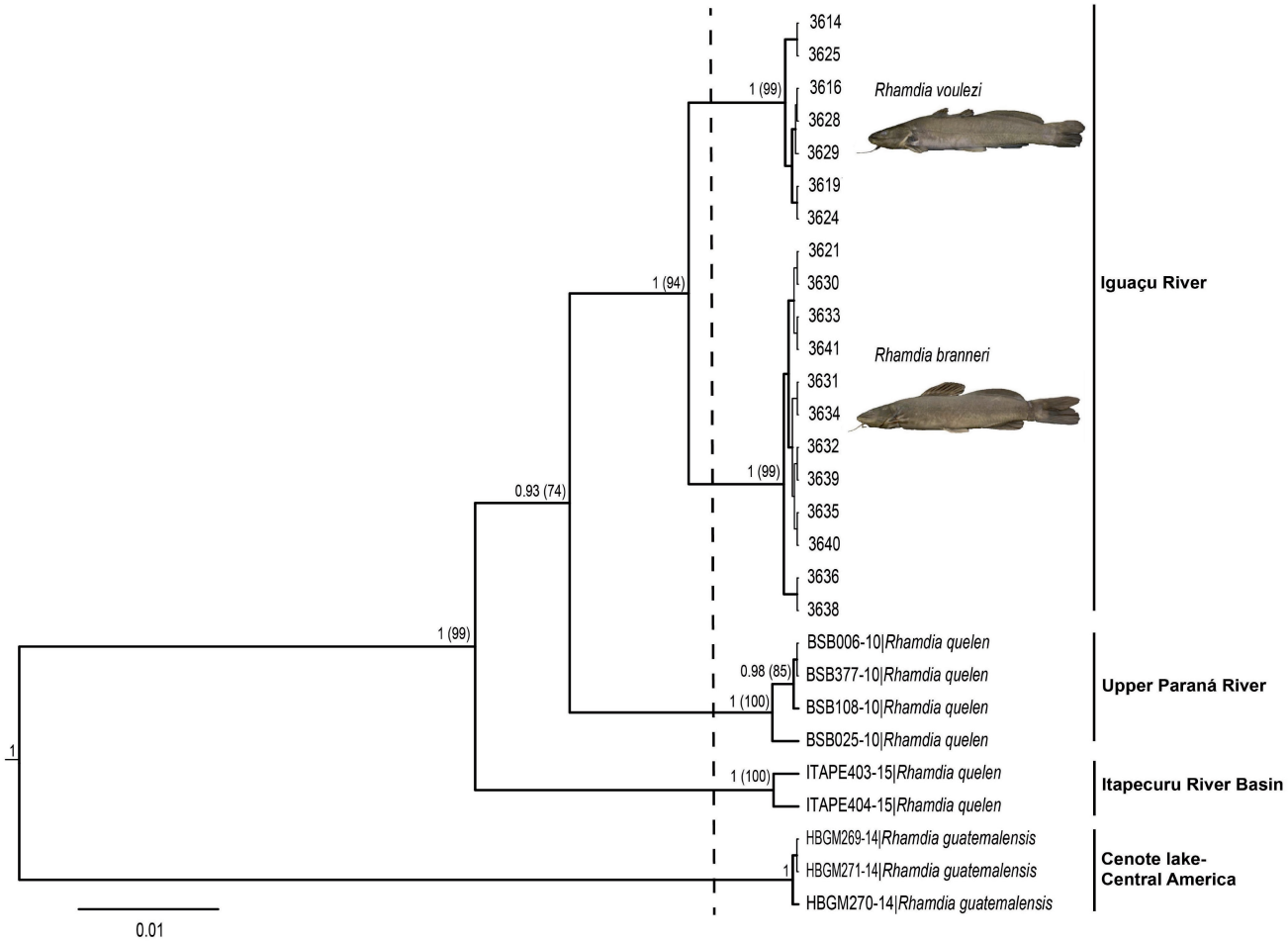

Fig. 3. The Bayesian inference tree obtained using the COI gene in BEAST v1.8.3. The numbers above the branches are the probability values (posterior probability) for the Bayesian model and the values in parentheses represent the NJ-K2P model, with a bootstrap of 1000 replicates. The vertical line indicates the species delimitation assigned by the single-threshold method in GMYC analysis. Photos by Tiago Debona.

Tab. 3. Intra- (in bold) and inter-MOTU genetic distances using the COI gene and the K2P model (Optimized threshold value = 0.77%). Values are shown as percentages. [1]Sequences from the BOLD data system.

Species	1	2	3	4
1. *Rhamdia voulezi*	**0.0**			
2. *Rhamdia branneri*	1.4	**0.0**		
3. *Rhamdia quelen*[1]	3.2	3.9	**2.7**	
4. Rhamdia guatemalensis[1]	8.4	8.8	9.3	**0.0**

Discussion

DNA barcoding analysis identified *R. branneri* and *R. voulezi* as distinct MOTUs, corroborating with reappraisal suggested by Garavello, Shibatta (2016). According to these authors, *R. branneri* and *R. voulezi* have morphological characteristics that permit its discrimination and maintenance of the names, such as the morphology of the dorsal and adipose fins, maxillary barbels, morphometric characteristics and pigmentation. In addition, both species exhibited inter-MOTU divergences higher than OT in comparison with *R. quelen* (>3.2%, Tab. 3).

Inter-MOTU distances were assessed using a method that calculates specific OT values from the analyzed dataset. Originally, DNA barcoding analysis applied the cut-off threshold of 1% for the analyzed species delimitation (Ratnasingham, Hebert, 2007). In specific groups, such as the Neotropical fish, studies have used the barcoding threshold (approximately 2%) proposed by Ward (2009) to delimit a wide-range of species (Rosso *et al.*, 2012; Bellafronte *et al*,. 2013; Pereira *et al.*, 2013; Gomes *et al.*, 2015; Shimabukuro-Dias *et al.*, 2016; Díaz *et al.*, 2016). Nonetheless, given the great diversity of

species, particularly related to life histories and complexity of environment, a fixed threshold value can under- or overestimate the correct number of MOTUs (Brown *et al.*, 2012). The optimized threshold avoids pre-defined cut-off values, minimizing false positive or false negative errors (Collins *et al.*, 2012). When examining the sequences of the *Rhamdia* genus (Tab. 3), we found that if the cut-off value is used as suggested for Neotropical fish (2%), the molecular identification would be underestimated, with only four MOTUs. As the genetic distance between *R. branneri* and *R. voulezi* was 1.4% (Tab. 3), these two taxonomically distinct species should be considered a single MOTU. In this way, the OT calculated from our dataset allowed the correct identification of all five MOTUs, demonstrating its ability to discriminate these species from the target species in this study.

Taxonomic analysis of the *Rhamdia* genus is complex; the species present great similarity in morphological characteristics, making its identification more difficult and generating ample controversy. Originally described by Haseman (1911) as *R. branneri* and *R. branneri voulezi*, both species were reported as endemic from the Iguaçu River Basin. Some studies have had evidence to refute the taxonomic revision proposed by Silfvergrip (1996), where *R. voulezi* and *R. branneri* were considered synonyms of *R. quelen*. With regard to karyotype differentiation and the presence of chromosome B in *R. branneri* and *R. voulezi*, Abucarma, Martins-Santos (2001) found a range of chromosomes (58-62) that overlap in number for both species. However, *R. voulezi* and *R. branneri* can be differentiated by the morphology of chromosome B, which can be used as a specific marker (Abucarma, Martins-Santos, 2001).

Although there is overlap in the number of chromosomes, morphological studies support the evidence in considering *R. voulezi* and *R. branneri* as valid independent species (Garcia, 2009; Baumgartner *et al.*, 2012; Mise *et al.*, 2013). Recently, Garavello, Shibatta (2016) described a series of morphological characteristics that discriminate between *R. voulezi* and *R. branneri*, suggesting provision of the names for both. The method applied by Garavello, Shibatta (2016) and the DNA barcoding were congruent and conclusive in identifying *R. branneri* and *R. voulezi* as valid and separating both species from *R. quelen*.

Representatives of *R. branneri* and *R. voulezi* are cultivated and commercialized for fish-farms and fee-fishing farms only as jundiá (same common name used for *R. quelen*). The confirmation and validation of *R. branneri* and *R. voulezi*, based on both morphological characters (Garavello, Shibatta, 2016) and molecular data, contribute to the conservation of the genetic resources of these species, preventing genetic contamination for the production of unintended hybrids. According to Hashimoto *et al.*, (2016), the negative impacts resultant from the erroneous use of hybrids in the genetic integrity of wild pure species are not considered by fish farmers. Problems related to wild populations produced for inappropriate crosses can also be damaging to aquaculture activity (Belle, Nash, 2009). In addition, the correct identification of the species contributes to genetic breeding and the integrity of natural stocks (Hashimoto *et al.*, 2011).

DNA barcoding analysis corroborates the morphological (Shibatta, Garavello, 1995; Garavello, Shibatta, 2016), karyotypic (Abucarma, Martins-Santos, 2001; Moraes *et al.*, 2007; Garcia *et al.*, 2003; Garcia *et al.*, 2010; Martinez *et al.*, 2011) and ecomorphological studies (Mise *et al.*, 2013), confirming *R. branneri* and *R. voulezi* as valid species. The correct definition of MOTUs and definition of the species is essential to the conservation of wild stocks and to minimizing the possibility of species translocations through aquaculture activity. At last, we strongly indicate the molecular and taxonomic analysis of *Rhamdia quelen*, which, due to the high level of intraspecific divergences in the analyzed data, shows cryptic genetic diversity that needs to be thoroughly evaluated.

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