

Rapid Communication

Molecular analysis confirms the introduction of a second species of yellow peacock cichlid *Cichla monoculus* Spix & Agassiz 1831 (Cichliformes: Cichlidae) in the Southeast Atlantic Hydrographic province, Brazil

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Abstract

Cichla kelberi, *Cichla monoculus*, and *Cichla ocellaris* are recognised as the most widespread introduced species of yellow peacock cichlids but most studies have failed to accurately identify these species in the invaded systems. The present study used molecular analysis to firstly confirm the introduction of *C. monoculus* in the Southeast Atlantic Hydrographic province, which encompasses areas from the three most populated Brazilian states. The cytochrome *b* sequences of 13 yellow peacock cichlids captured at two reservoirs from Paraíba do Sul river basin confirmed six of them as *C. monoculus* and seven as *C. kelberi*. Phylogenetic analysis revealed that the six *C. monoculus* caught in the Paraíba do Sul river clustered in a well-supported clade with other *C. monoculus* sequences available in GenBank. Some individuals showed divergences in colouration pattern from that of the original description of *C. monoculus*, suggesting that this species could be interbreeding with *C. kelberi*. Our findings stressed the applied importance of using molecular approaches to accurately detect invasive cryptic species and to track the spread of yellow peacock cichlids outside their native range.

Key words: invasive species, alien fish, peacock bass, molecular analysis, cryptic species, Neotropical

Introduction

Peacock cichlids (*Cichla* spp.) are Neotropical piscivorous fish that have been introduced into several freshwater systems around the world (Santos et al. 2016). Among the 15 species of *Cichla* currently validated through morphotaxonomic studies (Eschmeyer et al. 2016), the yellow peacock cichlids are a group of five similar species that share most morphological and colouration features (Kullander and Ferreira 2006). Together with the blue peacock cichlid *Cichla piquiti* Kullander and Ferreira, 2006, three species of yellow peacock cichlids—*Cichla kelberi* Kullander and Ferreira, 2006, *Cichla monoculus* Spix & Agassiz, 1831, and *Cichla ocellaris* Bloch &

Schneider, 1801—are recognized as the major members of this widespread genus that has been introduced throughout the American continent (Espínola et al. 2015; Marques et al. 2016).

The yellow peacock cichlid *Cichla monoculus* Spix & Agassiz, 1831 occurs naturally in floodplain lakes of the Solimões, Negro, Trombetas and Amazon-Orinoco river basins, and is also found in Peru and Colombia (Kullander and Ferreira 2006; MZUSP 2016). Non-native populations have been recorded in several reservoirs in north eastern, south eastern, and southern Brazil (Chellappa et al. 2008; Almeida-Ferreira et al. 2011; Briñez et al. 2013), with probable adverse impacts on native fish assemblages, as is reported for its congeners (Santos et al.

2001; Latini and Petrere 2004). Although identification of *C. monoculus* within its native range is facilitated by its natural geographic segregation in relation to the other species of yellow peacock cichlids, this is not the case for non-native populations, particularly because of the chance of multiple introductions of different congeneric species.

Consequently, despite records since the early 1970s of non-native yellow peacock cichlid populations (Zaret and Paine 1973; Oliveira et al. 1986; Santos et al. 2001), few studies have accurately confirmed that *C. monoculus* was the actual species introduced into these systems. The continuous temporal changes in species identification of the yellow peacock cichlids introduced into Brazilian reservoirs provide an iconic example of how difficult the precise recognition of these species from non-native populations is. Until the 2000s all studies considered *C. ocellaris* as the single invasive species of yellow peacock cichlid introduced throughout Brazil (Peixoto 1982; Oliveira et al. 1986; Agostinho et al. 1994). By taking into account biogeographic distribution of peacock cichlids within their native range, studies published between 2001 and 2006 regarded *C. monoculus* as the main species of yellow peacock cichlid introduced outside the Amazon region, also switching the species name (i.e. replacing *C. ocellaris* by *C. monoculus*) of some previously known non-native populations (Araújo and Santos 2001; Santos et al. 2001; Chellappa et al. 2003; Latini and Petrere 2004; Novaes et al. 2004; Marques and Resende 2005). After Kullander and Ferreira's (2006) study, which described nine new species of peacock cichlids and reviewed the biogeographic distribution of all fifteen described species for this group, some known non-native populations of *C. monoculus* were renamed again as being *C. kelberi* (Santos et al. 2008; Santos et al. 2011; Fragoso-Moura et al. 2016) or *C. piquiti* (Resende et al. 2008), or even changed back to *C. ocellaris* (Espinola et al. 2015). Only a few studies using molecular approaches confirmed the presence of *C. monoculus* outside its native range, all of which non-native populations were recorded for reservoirs and a floodplain system in the Upper Paraná river basin (Oliveira et al. 2006; Almeida-Ferreira et al. 2011; Briñez et al. 2013).

Genetic analyses using mitochondrial DNA are valuable tools to evaluate population structure, phylogenetic distance, and evolutionary relationships among individuals, populations, and species (Avice 2004). Mitochondrial DNA presents unique characteristics such as maternal transmission, absence of recombination, and also evolves 5–10 times faster than nuclear DNA, so is extremely helpful in genetic analysis of fish populations (Avice 2004; Mabuchi et al. 2008;

Marques et al. 2016). The mtDNA marker, cytochrome *b* (*cytb*) undergoes slow levels of gene evolution, and has more variable regions (Farias et al. 2001). Moreover, *cytb* has been shown to be useful, either alone or in combination, for assessing relationships among Cichlidae species, especially those of the genus *Cichla* (Farias et al. 2001; Willis et al. 2007, 2012).

In the present study, molecular analyses performed on yellow peacock cichlids captured through an assessment programme for invasive fish species confirmed the introduction of *C. monoculus* at the Paraíba do Sul river, one of largest and most important Brazilian rivers. This is the first record of a second species of yellow peacock cichlid introduced into the Southeast Atlantic Hydrographic province (~ 230,000 km²), which encompasses areas from the three most populated states in Brazil. In addition to addressing the potential use of molecular approaches for disentangling the identification of invasive fish species of very similar morphological characteristics, such as the case of the yellow peacock cichlids, the major implications of the introduction of *C. monoculus* at local and regional scales are discussed.

Material and methods

Study site

Paraíba do Sul river, the largest and the most important river in the Southeast Atlantic Hydrographic province (Pinto et al. 2008), is born at the confluence of the Paraitinga and Paraibuna rivers in São Paulo State. Paraibuna reservoir (23°22'04.4"S; 45°39'27.4"W; Figure 1) is a 177 km² mesotrophic impoundment located at the most upstream portion of the Paraíba do Sul river. Situated at 715 m above mean sea level (a.m.s.l.), this reservoir was built in 1978 to regulate the water flow of the Paraíba do Sul river and insure a water supply to the population of Rio de Janeiro State (Pinto et al. 2008). Ilha dos Pombos reservoir is a 4.3 km² run-of-the-river impoundment (21°50'40"S; 42°34'45.1"W) with 32 m maximum depth and 12 m average depth (Aguiar 2008). This reservoir was built in 1924 and still ranks as the most downstream reservoir of the Paraíba do Sul river mainstem. It is located at 135 m a.m.s.l., and shows fair levels of water quality despite the trend of increasing sewage pollution (Brito et al. 2012).

Sampling and morphological data analysis

Samples of yellow peacock cichlids were obtained by hook and line (using artificial baits) performed

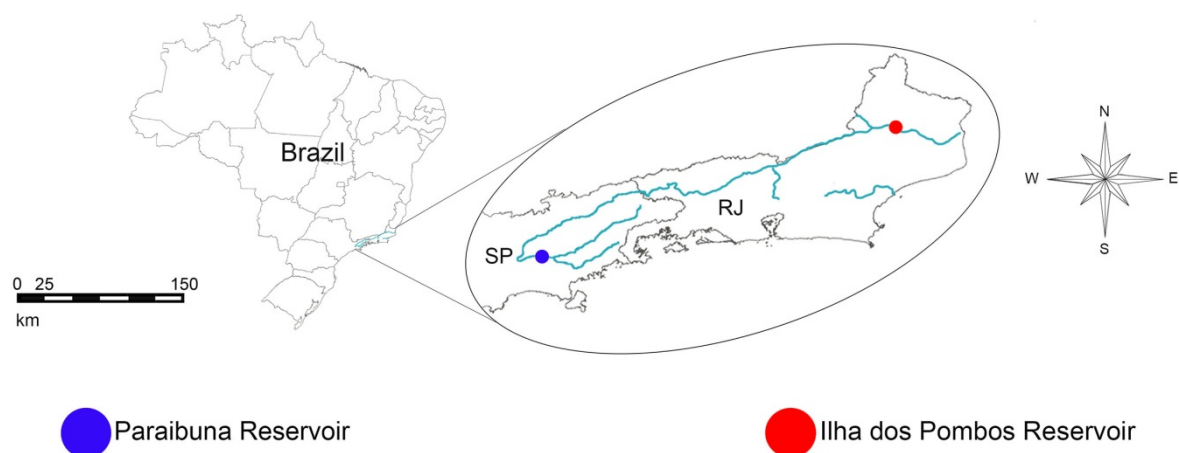


Figure 1. Geographic location of the Paraibuna and Ilha dos Pombos reservoirs, which are located, respectively, in the upper and lower stretches of the Paraiba do Sul river mainstem. SP = Sao Paulo State; RJ = Rio de Janeiro State

over four days (ca. 28 hours of angling) from April to June 2016 in Paraibuna and Ilha dos Pombos reservoirs, Paraíba do Sul river basin. All specimens were euthanized on ice, measured (total and standard length; mm), and weighed (g). The following morphological traits were measured (mm), for all individuals of peacock cichlids, according to Kullander and Ferreira (2006): head length, head depth, body depth, orbit diameter, caudal peduncle depth, caudal peduncle length, maximum body depth, and maximum body width.

DNA extraction, amplification and sequencing

Peacock cichlids were dissected and kept frozen at -20°C until DNA extraction. In order to extract total genomic DNA, the NucleoSpin Tissue kit (Macherey-Nigel GmbH & Co. KG) was used following the manufacturer's instructions and the products of extraction were stored at -20°C . The mitochondrial molecular marker cytochrome *b* (*cytb*) was used to infer genetic relationships among the samples obtained in the present study and other sequences of *Cichla* available in GenBank. The *cytb* region was amplified via PCR with the primers *Cytb3-3'* (3'-GCCAAATAGGAARTATCATTC-5') and *GLUDG-5'* (5'-CGAAGCTTGACTTGAARAA CCAAYCGTTG-3') (Lee et al. 1995; Palumbi 1996). This region codes for a protein product that is involved in mitochondrial respiration and is constrained in its mutation pattern (Willis et al. 2007). The reaction mixture (25 μL) contained 1 unit (U) Taq DNA polymerase (ThermoScientific Inc., USA), $1\times$ reaction buffer with NH_4SO_4 , 2.5 mM MgCl_2 ,

0.16 mM dNTPs (ThermoScientific Inc., USA), 8 pmol of each primer and 5 ng of genomic DNA. The amplification of *cytb* were successful with the following conditions: 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 45°C for 30 sec, 72°C for 15 sec, and a final extension at 72°C for 5 min. PCR products were purified and sequenced by MacroGen Inc. (Seoul, Korea).

Genetic analysis

The sequences obtained in the present study were analysed together with other *C. kelberi*, *C. monoculus* and *C. ocellaris* sequences retrieved from GenBank corresponding to the clade B1 proposed by Willis et al. (2012) ("*Cichla ocellaris* clade"). Up to 15 sequences from each species were included in the analysis. *Retroculus* sp. sequences were used as an outgroup. Sequences were aligned using MAFFT v7 (Kato and Standley 2013). The MEGA v6.06 software (Tamura et al. 2013) was used to select the best fit model of nucleotide substitution (HKY+G) and to construct Maximum Likelihood (ML) phylogenetic trees with 1000 bootstrap replications. For a better comparison between different studies, the intraspecific and interspecific p-distances were also calculated using MEGA.

The phylogenetic relationships between *Cichla* species were also examined using Bayesian Inference (BI) with MrBayes v3.2 (Ronquist et al. 2012). To sample across nucleotide substitution models, the command "lset nst = mixed" was used before running the analysis. The Markov Chain Monte Carlo procedure consisted of two independent trials with four

Table 1. Morphometric measurements of *Cichla monoculus* (mean; min-max) captured at Paraibuna and Ilha dos Pombos reservoirs, Paraíba do Sul river basin, and comparisons with those used for morphotaxonomical description (Kullander and Ferreira 2006). Measurements are in percent of SL, except SL, mouth depth, and mouth width (mm).

Traits	Present study (N=6)	Kullander and Ferreira (2006) (N=35)
SL (mm)	232.2 (195 – 306)	200.5 (44.3 – 343.9)
Head length (%)	32.4 (30.1 – 34.0)	33.5 (31.5 – 35.3)
Head depth (%)	18.9 (18.4 – 19.3)	24.1 (21.2 – 29.7)
Body depth (%)	27.2 (25.0 – 29.6)	30.6 (27.1 – 34.6)
Orbit diameter (%)	5.9 (4.8 – 6.7)	8.5 (6.8 – 11.3)
Caudal peduncle depth (%)	11.1 (9.6 – 12.2)	11.1 (9.9 – 12.6)
Caudal peduncle length (%)	15.4 (10.7 – 17.4)	18.3 (15.2 – 20.2)
Caudal peduncle width (%)	7.0 (6.2 – 7.4)	
Maximum body width (%)	13.8 (12.8 – 14.4)	
Mouth depth (mm)	16.6 (15.0 – 17.8)	
Mouth width (mm)	20.0 (18.2 – 22.0)	

chains each. Each chain was run for 2,000,000 generations and sampled every 100th cycle. Posterior probability (PB) values for the resulting 50% majority rule consensus tree were estimated after discarding the first 25% of trees as burn-in.

Results

A total of 13 yellow peacock cichlids were caught through angling, with six individuals being identified as *C. monoculus* (three from Ilha dos Pombos and three from Paraibuna reservoirs: KX954330.1–KX954335.1), and seven as *C. kelberi* (two from Ilha dos Pombos and five from Paraibuna). All six *C. monoculus* specimens showed three dark vertical bars, body colour from yellow to greenish with light spots sometimes covering the body, and black or ocellated stripes or spots absent on the head or on the gill (Figure 2). Some individuals showed irregular dark blotches on the anterior abdominal sides (Figure 2 B). Light spots on both pelvic and anal fins were recorded for at least one *C. monoculus* individual, differing from the original description of *C. monoculus* provided by Kullander and Ferreira (2006). Overall, the morphological measurements obtained for all six *C. monoculus* captured in the Paraíba do Sul river (Table 1) agreed well with the values provided by Kullander and Ferreira (2006) for the individuals used for species description. Slight deviations observed for some measurement proportions are probably related to differences between the size range of *C. monoculus* used in this study and that used by Kullander and Ferreira (2006).

The length of the PCR products obtained via 1.0 % agarose gel were ~650 bp. Multiple sequence alignment originated a matrix containing 655 sites. We identified only one haplotype for *C. monoculus* individuals in



Figure 2. Photographs of two *Cichla monoculus* captured in the Paraíba do Sul river basin, showing the variations in colour patterns: A–250 mm TL (from Paraibuna Reservoir); B–370 mm TL (from Ilha dos Pombos Reservoir). Photo by by Ana Clara Franco.

all sampled reservoirs. The Maximum Likelihood and Bayesian analysis demonstrated similar results. The phylogenetic analysis based on the *cytb* region revealed that the six specimens assigned as *C. monoculus* grouped in a well-supported clade with another seven *C. monoculus* sequences available in GenBank (bootstrap = 81% and PB = 1.0). A couple of *C. ocellaris* sequences (NC030272.1 and KU878410.1) retrieved from GenBank also clustered with these *C. monoculus* sequences (Figure 3). Additionally, the two samples from Ilha dos Pombos (KX954328.1 and KX954329.1) and five samples from Paraibuna reservoirs (KX954323.1 – KX954327.1)

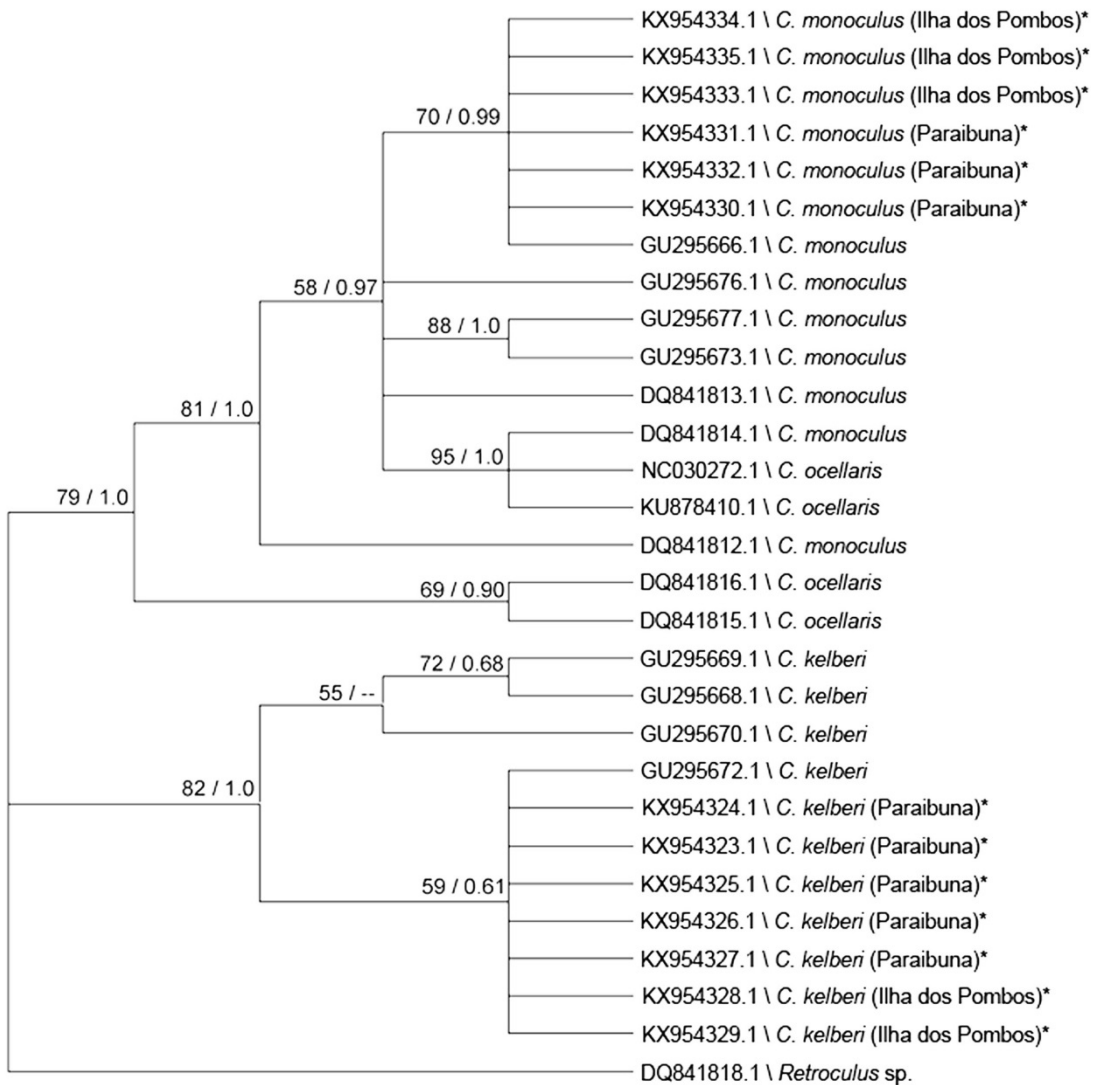


Figure 3. Phylogenetic tree presenting consensus topology from Bayesian Inference (BI) and Maximum Likelihood (ML) analyses based on Cytochrome *b* (*cytb*) sequences of *Cichla* species. Supports at nodes are bootstrap values from Maximum Likelihood analysis and posterior probability from Bayesian inference, respectively (cut-off = 50%). Strains are identified by their GenBank accession number and species name. (Name of the reservoir)* = New sequences published in the present study.

identified as *C. kelberi* in the present study formed a monophyletic clade with another four *C. kelberi* sequences available in GenBank (bootstrap = 82% and PB = 1.0). The p-distance between *C. monoculus* and *C. kelberi* was slightly higher (1.6%) than the average p-distance among the three species of yellow peacock cichlids (1.2%). The mean p-distance within *Cichla* spp. evaluated in this study was 0.7%. Therefore, the interspecific molecular variation was approximately 1.7 times higher than the mean genetic divergence within species.

Discussion

The present study is the first to validate through molecular analysis the presence of non-native *C. monoculus* populations in the Southeast Atlantic Hydrographic province, of which Doce and Paraíba do Sul are the most important rivers. Our findings also confirmed through molecular analysis the presence of *C. kelberi* in Paraibuna and Ilha dos Pombos reservoirs, but this result was not so impressive since Marques et al. (2016) have detected

non-native populations of *C. kelberi* in six reservoirs located within the middle stretch of the Paraíba do Sul river. Although *C. monoculus* was previously assigned as the yellow peacock cichlid species introduced into the Doce river (Latini and Petrere 2004), there is no molecular sequence available for *C. monoculus* from this river in GenBank or even museum records for the presence of this species in non-native systems except the Paraná river basin (MZUSP 2016). In addition, Fragoso-Moura et al. (2016) apparently reassigned the yellow peacock cichlid species introduced into the Doce river as being *C. kelberi*. Therefore, our study not only reports for the first time the presence of *C. monoculus* in the Paraíba do Sul river mainstem, but confirms that this yellow peacock cichlid was introduced in other large river basins beyond the Paraná river basin (Oliveira et al. 2006; Briñez et al. 2013; Gasques et al. 2015). Our findings also highlight the importance of molecular markers as tools for accurate identification of invasive fish species, especially in the case of non-native populations lacking biogeographical information to support species classification.

Five species of yellow peacock cichlids (i.e. *C. kelberi*, *C. monoculus*, *C. pleiozona*, *C. nigromaculata* and *C. ocellaris*) are recognised as valid species through morphotaxonomic approaches (Kullander and Ferreira 2006). However, the alternative hypothesis supported by molecular analyses argues for the existence of only one single species of yellow peacock cichlid (*Cichla ocellaris sensu lato*) in which multiple morphotypes are affected by occasional intraspecific gene exchange (Willis et al. 2012). While this issue is still under debate, both morphotaxonomic and molecular studies agree that *C. monoculus* and *C. kelberi* do not share the same geographic distribution in their native ecosystems (Kullander and Ferreira 2006; Willis et al. 2012). By detecting the co-occurrence of non-native populations of *C. monoculus* and *C. kelberi* in two reservoirs located at the most upstream and downstream stretches of the Paraíba do Sul river mainstem, our findings demonstrated that at least two introduction events of yellow peacock cichlid occurred in this system. Recurrent events of introduction are of great ecological concern, since they can confound studies tracking the spread of invasive populations and because continuous events of introduction can contribute to keeping non-native populations at high densities, harshening impacts over native fish fauna (Kolbe et al. 2004; Carvalho et al. 2014; Hamilton and Miller 2016; Pfennig et al. 2016).

The potential for hybridisation among introduced peacock cichlid species is another important subject

that arises from our findings. Willis et al. (2012) suggested the occurrence of variable levels of introgressive hybridisation among most *Cichla* species within their native range, as previously detected for *C. monoculus* and *C. temensis* in the Amazon river basin (Teixeira and Oliveira 2005). Hybridisation was also detected among invasive populations of yellow peacock cichlid *C. kelberi* and blue peacock cichlid *C. piquiti* in the Paraná river basin (Almeida-Ferreira et al. 2011). Taking into account that *C. monoculus* and *C. kelberi* are apparently co-occurring in many reservoirs and other systems within Paraíba do Sul river basin (Marques et al. 2016; present study), hybridisation between these two close species of yellow peacock cichlids is expected to occur. In fact, our findings suggest that the process of hybridisation between *C. monoculus* and *C. kelberi* is already on course, since the light spots we observed on both anal and pelvic fins for some *C. monoculus* are not expected to be found in this species according to its taxonomic description. However, these same light spots are considered as key diagnostic features for *C. kelberi* which can usually be used to differentiate it from *C. monoculus* (Kullander and Ferreira 2006).

In conclusion, our study was the first to use molecular analyses to validate the presence of non-native *C. monoculus* populations in the largest and most important river basin within the Southeast Atlantic province. This is quite a surprising result, since this species has been reported for a long time in the literature as one of the major yellow peacock cichlids introduced into freshwaters outside the Amazon. Detection of *C. monoculus* in systems where another species of peacock cichlid is already present might wrongly appear as a matter of minor importance, because adverse ecological interactions with native fish assemblages have already been triggered. However, the presence of a second non-native species of yellow peacock cichlid can obscure the detection of recurrent introductions; make it difficult to track the spread pattern and invasion pathways of non-native populations; and contribute to increasing the genetic variability and fish abundance of introduced species through intra- and interspecific hybridisation. Therefore, our findings highlight the applied importance of using molecular markers to accurately detect the presence of invasive fish species of very similar morphological and colouration features but that have different evolutionary trajectories (i.e. cryptic species). This approach is also useful to track the spread of yellow peacock cichlids and to detect recurrent and imperceptible stocking of these species outside their native range.

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