

Genetic Structure of the Mangrove Killifish *Kryptolebias hermaphroditus* Costa, 2011 (Cyprinodontiformes: Aplocheiloidei) Supports A Wide Connection among its Populations

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The *Kryptolebias marmoratus* species group is composed of the only three vertebrate species that lack females. These species present only males and simultaneously hermaphroditic individuals; that are able to reproduce by allogamy, with males, or by autogamy, performing self-fertilization and generating clones of themselves. The proportion of males is variable among those species and even among their populations. *Kryptolebias hermaphroditus* has the smallest proportion of males. Indeed, no males have been recorded in most known populations. This is a mainly autogamous species, with small populations having a disjunct distribution along the eastern and northern coast of Brazil. Species presenting such adaptations would be expected to have an elevated rate of genetic population structure, reflecting any barriers that obstruct gene flow between populations. Partial sequences of the mitochondrial cytochrome c oxidase I (COI) gene from 335 individuals were sampled to perform a population analysis. Only a single haplotype of COI, widely distributed throughout all the sampled populations, was recovered for *K. hermaphroditus*. Here we hypothesize that the high degree of communication within populations is probably the main biological feature leading to this pattern.

Key words: DNA-barcoding, Gene flow, Mitochondrial DNA, Neotropical, Self-fertilizing hermaphrodites, South America.

BACKGROUND

Fish species inhabiting mangroves and other brackish water environments present physiological adaptations to tolerate rapid daily variation in temperature, salinity, pH and oxygen availability (Evans et al. 1999; Cooper et al. 2013). The Atlantic

coast of the American continent has a number of habitats with these characteristics and within them there is a lineage of fishes considered to be major model organisms for different research areas, such as behavioural, ecotoxicogenomics, embryology, genetics, molecular carcinogenesis, and physiology: the *Kryptolebias marmoratus* species group (Hsu et

al. 2006; Lee et al. 2008; Earley and Hsu 2008; Costa 2011; Avise and Tatarenkov 2015). The Aplocheiloidei genus *Kryptolebias* Costa, 2004 includes both species occurring exclusively in freshwater environments, as well as species in the *K. marmoratus* group, which are unique to estuarine habitats (Harrington 1961; King et al. 1989; Davis et al. 1990; Costa 2004). Species in this group, popularly known as mangrove killifishes, are distributed from the south-eastern United States to southern Brazil, including the Caribbean Islands, but with distribution gaps along the Brazilian coast (Costa 2004 2006 2008 2011; Lira et al. 2015 2021; Berbel-Filho et al. 2016; Guimarães-Costa et al. 2017; Tatarenkov et al. 2017a). The *K. marmoratus* group constitutes a well corroborated clade supported by both morphological and molecular data (Tatarenkov et

al. 2009; Costa et al. 2010; Costa 2011 2016; Berbel-Filho et al. 2016), and comprises three species: *K. marmoratus* (Poey, 1880), occurring from Florida to northern Brazil, including records in the Caribbean Islands (Tatarenkov et al. 2011; Lira et al. 2021); *K. ocellatus* (Hensel, 1868), inhabiting coastal basins of eastern to southern Brazil (Costa 2016; Berbel-Filho et al. 2020); and *K. hermaphroditus* Costa, 2011 (Fig. 1), found in coastal environments from eastern to northern Brazil (Costa 2011 2016; Berbel-Filho et al. 2016; Guimarães-Costa et al. 2017). These species are the only Neotropical members of Aplocheiloidei that inhabit brackish, and eventually marine waters (Cooper et al. 2013; Costa 2016).

Besides physiological adaptations to abiotic disturbances, the three species in the *K. marmoratus*



Fig. 1. *Kryptolebias hermaphroditus* from Tutóia, Maranhão State, Delta do Parnaíba, north eastern Brazil; UFRJ12666: A: Hermaphrodite, 35.5 mm SL; B: Male, 20.3 mm SL; C: Male, 28.9 mm SL.

group have an unusual adaptation that is unique among vertebrates: no females are present. Instead, the three species have simultaneous hermaphrodites, which may act as females when reproducing with males but are also able to perform self-fertilization, consequently producing clone offspring (Harrington and Kallman 1968; Taylor 2001; Costa 2006 2011 2016; Mackiewicz et al. 2006; Costa et al. 2010; Berbel-Filho et al. 2016). Males are present in all three species; however, the proportions may differ significantly between each lineage. In *K. ocellatus*, males account for about 50% of the population (Costa et al. 2010; Costa 2016). In *K. marmoratus* males have been recorded in all known populations, but their frequency is variable, with males being more common within populations in Belize (corresponding to 25% of the population), or rarer, as recorded within populations in Florida (representing less than 1% of the recorded individuals) (Kristensen 1970; Davis et al. 1990; Turner et al. 1992; Taylor

2001; Mackiewicz et al. 2006). For *K. hermaphroditus*, males have never been recorded in most of its known populations. This species was described by Costa (2011) based only on hermaphrodite individuals from estuarine areas of coastal rivers and lagoons of south-eastern Brazil, in Rio de Janeiro and São Paulo states. Despite the presence of several individuals in different collections no males were detected for this species, and therefore it was first proposed that *K. hermaphroditus* included only self-fertilizing hermaphrodites (Costa 2011).

In the last ten years, extensive collection efforts along the coastal areas of south-eastern, north-eastern and northern Brazil have provided better knowledge on the distributional range of *K. hermaphroditus*. Currently, more than 30 populations have been recorded with major geographical gaps between most of them (Fig. 2) (Costa 2011 2016; Sarmento-Soares et al. 2014; Lira et al. 2015 2021; Berbel-Filho et al. 2016; Guimarães-

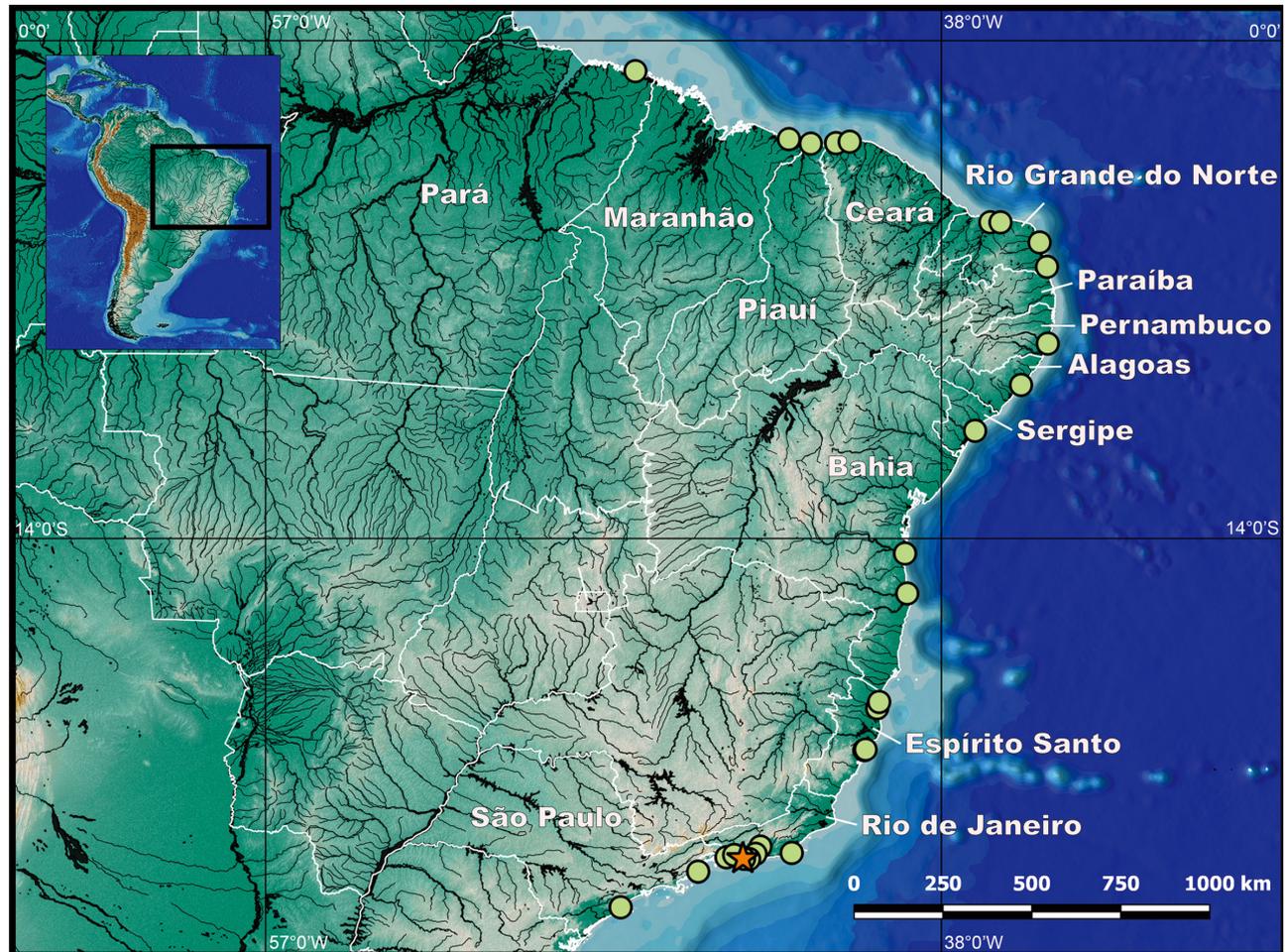


Fig. 2. Distribution of *K. hermaphroditus*: Orange star indicates type locality; and Green circles indicate recorded localities for the species (Costa 2011; 2016; Sarmento-Soares et al. 2014; Lira et al. 2015; Berbel-Filho et al. 2016; Guimarães-Costa et al. 2017; Tataronov et al. 2017a; This study).

Costa et al. 2017; Tatarenkov et al. 2017a; this study). Regardless of the increase in the number of recorded populations, in most cases only few individuals were collected in each locality and the vast majority of them were hermaphrodites. So far, only eight males have been found in just four populations (Berbel-Filho et al. 2016; Costa 2016; this study). The scarcity of males recorded for *K. hermaphroditus* suggests that the reproduction in this species is mainly regulated by self-fertilization (Mackiewicz et al. 2006; Costa et al. 2010; Costa 2011 2016). Species with a high degree of autogamy present a reduced genetic population size due to inbreeding (Charlesworth and Wright 2001). Therefore, it is expected that the population structure of autogamous species are stronger than in allogamous species (Charlesworth 2003), specially when geographic barriers or long distances could restrict the gene flow between populations (Hardy and Vekemans 1999). In other words, when a population is geographically isolated, the probability that new haplotypes become fixed in this isolated population is higher in autogamous species than in allogamous species (Hardy and Vekemans 1999). In addition, several studies on different cyprinodontiforms lineages have corroborated the importance of mitochondrial genetic data to recover the intraspecific population structure in this group, even in cases of recent events of colonization due to human introduction (e.g., Costa and Amorim 2014; Berbel-Filho et al. 2018; Bragança et al. 2019).

Since *K. hermaphroditus* is mainly an autogamous species with disjunct population distributions, comprising only a few individuals, a high rate of population structuring is expected (Charlesworth 2003). Further, the lack of connectivity and gene flow between populations or groups of populations due to isolation by geographic barriers or long distances should also lead to population structuring (Hardy and Vekemans 1999). Thus, considering the expected population genetic pattern, the aim of the present study is to perform molecular analyses to understand how populations of *K. hermaphroditus* are genetically structured, and which factors could be affecting the distribution and genetic flow among their populations.

MATERIALS AND METHODS

Molecular analyses was performed on individuals of the three species of *Kryptolebias marmoratus* species group (*K. marmoratus*, *K. ocellatus* and *K. hermaphroditus*), comprising 321 previously published sequences of the mitochondrial gene cytochrome *c* oxidase I (*COI*) (Berbel-Filho et al. 2018 2020; Costa et al. 2017; Kim et al. 2016; Lara et al. 2010; Lee et

al. 2001; Tatarenkov et al. 2017a b 2018; Weigt et al. 2012), as well as 14 new sequences (Table S1). Populations encompassing most of the distribution area of all three species were sampled. The list of species, localities and the accession numbers of the 335 sampled sequences for the present study is available in the supplementary material (Table S1).

Genomic DNA was extracted from muscle tissue on the right side of the caudal peduncle using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer instructions. The DNA amplifications were performed using the primers LCO1490 and HCO2198 (Folmer et al. 1994) for the mitochondrial gene *COI*. Polymerase chain reaction (PCR) was performed in 15 µl reaction volumes containing 5× Green GoTaq Reaction Buffer (Promega), 3.2 mM MgCl₂, 1 µM of each primer, 75 ng of total genomic DNA, 0.2 mM of each deoxynucleotide triphosphate (dNTP) and 1U of Taq polymerase. The thermocycling profile was: (1) 1 cycle of 4 minutes at 94°C; (2) 35 cycles of 1 minute at 92°C, 1 minute at 47°C and 1 minute at 72°C; and (3) 1 cycle of 4 minutes at 72°C. In all PCR reactions, negative controls without DNA were used to check for contamination. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing reactions were made using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 10 µl reaction volumes containing 1 µl BigDye 2.5, 1.55 µl 5× sequencing buffer (Applied Biosystems), 2 µl of the amplified products (10–40 ng) and 2 µl primer. The thermocycling profile was set to 35 cycles of 10 seconds at 96°C, 5 seconds at 54°C and 4 minutes at 60°C. The sequencing reactions were purified and denatured and the samples were run on an ABI 3130 genetic analyzer (Applied Biosystems). For each PCR product, both strands were sequenced to generate the analysed sequences. Sequences were edited using the program MEGA X (Kumar et al. 2018) and aligned by ClustalW (Chenna et al. 2003).

The haplotype network analysis was performed based on a matrix including 335 individuals: 129 sequences of *K. ocellatus*; 117 of *K. marmoratus*; and 89 of *K. hermaphroditus* (Table S1). After aligning and trimming the sequences, the dataset included a total of 655 base pairs after aligned. The Median-joining network approach was executed in the program PopART 1.7 (Leigh and Bryant 2015).

RESULTS

The haplotype network (Fig. 3) recovered four main clusters corresponding to *Kryptolebias ocellatus*,

K. hermaphroditus, and the Central and Northern clades of *K. marmoratus*, *sensu* Tatarenkov et al. (2017a). For *K. ocellatus* 12 different haplotypes were recorded; for *K. marmoratus* 12 haplotypes were also recovered, of which five are from the Central clade and seven from Northern clade *sensu* Tatarenkov et al. (2017a). All individuals of *K. hermaphroditus* formed a single cluster represented by a single haplotype throughout the entire distribution of the species (Fig. 2).

DISCUSSION

The mangrove killifish *Kryptolebias hermaphroditus* is an euryhaline species present across a wide gradient of environmental conditions (Evans et al. 1999; Cooper et al. 2013). Thus barriers that could restrict gene flow between populations are probably related to marine habitats. Several fish species inhabiting a similar area to that of *K. hermaphroditus* usually present distributions that are limited by marine biogeographic ecoregions (*e.g.*, Spalding et al. 2007; Andrades et al. 2018). However, the current analyses recovered a single haplotype widely distributed throughout more than 3,500 km along the coastline (Figs. 2 and 3). This result not only contrasts with those found on the other

species of the *K. marmoratus* species group, in which several distinct haplotypes were recovered in different populations (Fig. 3) (Tatarenkov et al. 2009; Berbel-Filho et al. 2020), but is also the opposite of what is expected for a species with small disjunctly distributed populations which reproduce mainly by autogamy (Hardy and Vekemans 1999; Charlesworth and Wright 2001; Charlesworth 2003). In a recent study, Lira et al. (2021) proposed that the current distribution of *K. hermaphroditus* would be associated with the expansion of the area occupied by the mangrove tree *Rhizophora mangle* L. in the end of the last glacial era. However, *K. hermaphroditus* is not exclusively found in mangrove habitats; it is also frequently found in coastal canals, swamps and lagoons (*e.g.*, Costa 2011; Sarmiento-Soares et al. 2014). So, if the current distribution of *K. hermaphroditus* is the result of a recent expansion, it is probably not associated only with the historical contractions and expansions in the distribution range of the mangrove tree. Thus, here we hypothesize that the current geographic pattern of the species is probably due to the high degree of communication between populations.

Considering the small size of *K. hermaphroditus* individuals, and that this is a brackish water species inhabiting shallow environments (Costa 2011 2016), it

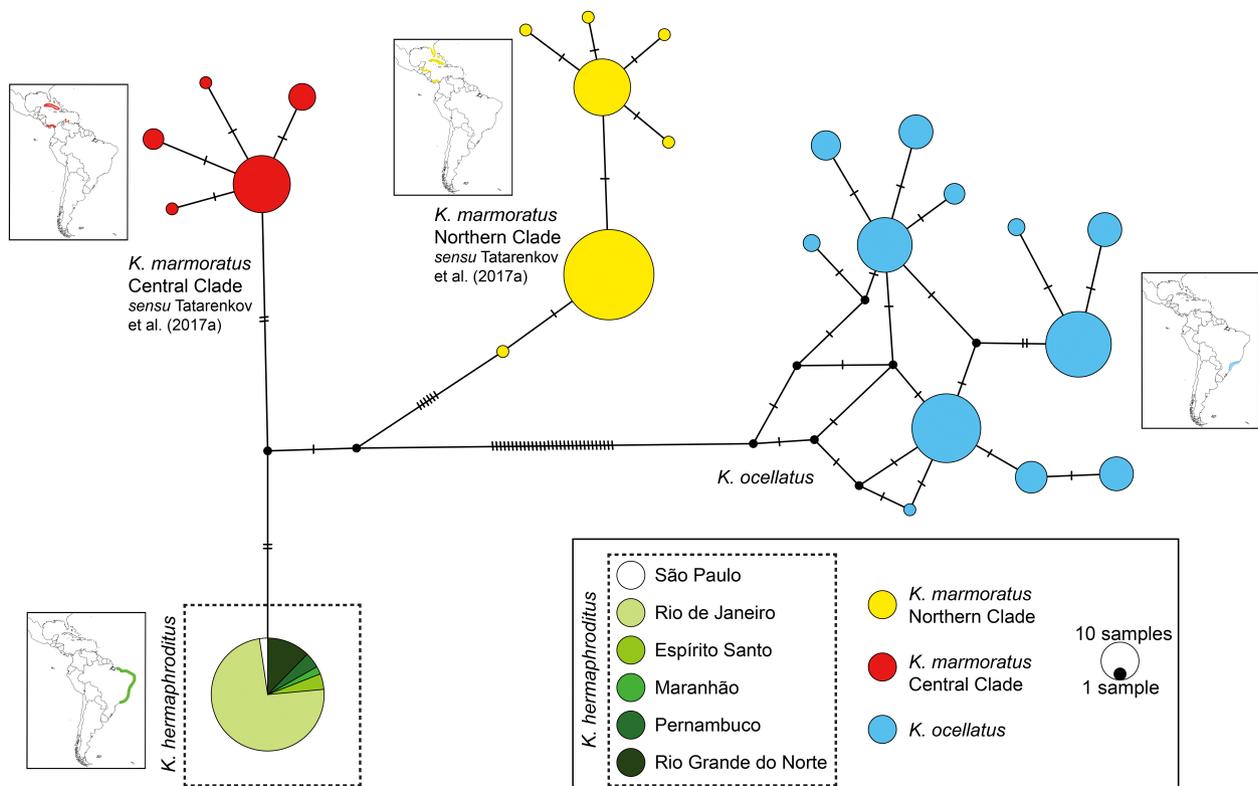


Fig. 3. Haplotype network of the *Kryptolebias marmoratus* species group. Maps represent the distribution of each group.

is unlikely that individuals are able to swim across the entire distribution of the species (Fig. 2). Therefore, the population communication corroborated by the results is probably maintained by interactions between neighbouring populations along the entire distribution of the species. If there were some discrete barriers obstructing the genetic flow of the species, or if the distance was a factor favouring the isolation between populations, then we would expect some population structuring to be recovered and show different haplotypes to be geographically associated to specific populations or group of populations (Hardy and Vekemans 1999; Charlesworth 2003). Recent records of additional populations along the Brazilian coast (Lira et al. 2021) also support this current wide communication between close populations hypothesis, favouring genetic flow through the entire distribution of the species.

The disjunct distribution known for *K. hermaphroditus* (Fig. 2) may also be an artifact due to application of inappropriate sampling methods and/or the loss of natural environments. Despite, brackish water species being the focus of several studies (e.g., Cognetti and Maltagliati 2000; Corsi et al. 2003; Pérez-Ruzafa et al. 2018); most of the collection effort in these environments is geared towards species that are economically valuable to fisheries (e.g., Hemingway and Elliott 2002; Fischer et al. 2011). It is likely that small fish species with no economic importance, such as *K. hermaphroditus*, are not sampled in more fisheries-oriented surveys. Another possible reason that may have contributed to the present disjunct distribution of *K. hermaphroditus* is the loss of natural habitats. Brazilian coastal areas have been explored and deforested since the end of the 16th century, initially by activities such as wood extraction and production of salt, and more recently by urban expansion, pollution and aquaculture (Godoy and Lacerda 2015; Ferreira and Lacerda 2016; Ottoni et al. 2021). The destruction of these brackish water environments may have affected several populations of *K. hermaphroditus* so that only disjunct populations have remained. Regardless of the causes of the current disjunct distribution of *K. hermaphroditus*, our data supports an alternative explanation for the low genetic divergence other than a recent range expansion during the end of the last glacial period promoted by mangroves expansion as proposed by Lira et al. (2021). Thus, communication between close populations is probably the main biological factor responsible for the recovered pattern, in which only a single haplotype was found in all the sampled populations, spanning more than 3,500 km along the Brazilian coastline.

The scarcity of *K. hermaphroditus* males could also be a factor contributing for the low genetic diversity recovered for the species. The present analysis

recovered a greater number of haplotypes for the other species of the *K. marmoratus* group, in which males are more common and consequently the allogamous reproduction is more usual, than for *K. hermaphroditus* (Fig. 3). For *K. marmoratus* it was proposed that males have an important role in increasing population genetic variation in moments of environmental stress (Ellison et al. 2011 2012). Males of *K. hermaphroditus* possibly play the same role in population dynamics, and the low frequency of these individuals could also be a factor contributing to the low genetic diversity recovered. However, more studies are necessary to understand how a higher presence of males and the allogamous reproduction could affect the genetic diversity of the mitochondrial DNA.

CONCLUSIONS

The present analysis corroborates *K. hermaphroditus* as a widely distributed species that may keep genetic flow among its populations. This broad range may conceal the real conservation status of the species. Most populations of *K. hermaphroditus* inhabit coastal ecosystems highly affected by human activity (Ferreira and Lacerda 2016; de Oliveira-Filho et al. 2016). Therefore, despite this wide distribution, the conservation of *K. hermaphroditus* is directly dependent on the maintenance of its natural habitats. Thus, more studies focusing on the biology of *K. hermaphroditus* are crucial for a better understanding of the population dynamics and genetic mechanisms acting in this species.

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Authors' contributions: All the authors equally contributed to the present study.

Competing interests: PFA, AMK, FPO and PHNB declared that they have no conflict of interest.

Availability of data and materials: All the data used in the present study is available on the GenBank

database, as indicated in the Supplementary Material of the manuscript.

Ethics approval consent to participate:

Individuals collected for this study were euthanized with a buffered solution of MS-222 at a concentration of 250 mg l⁻¹, for a period of 10 min or more, until opercular movements completely ceased, following the methods for euthanasia approved by CEUA-CCS-UFRJ (Ethics Committee for Animal Use of Federal University of Rio de Janeiro; permit number: 01200-001568/2013-87). Molecular data were obtained from specimens that were euthanized, fixed and preserved in absolute ethanol.

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Supplementary Materials

Table S1. List of species, localities and accession numbers of the 335 sequences analysed in the present study. (download)