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Hibridização e introgressão entre *Cichla piquiti* e *Cichla ocellaris* var. *kelberi*: evidências
moleculares e morfométricas em ambientes nativo e não-nativo

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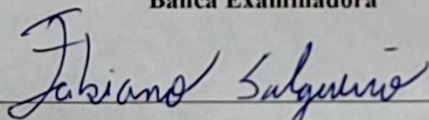
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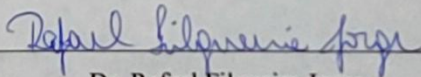
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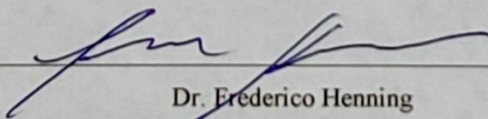
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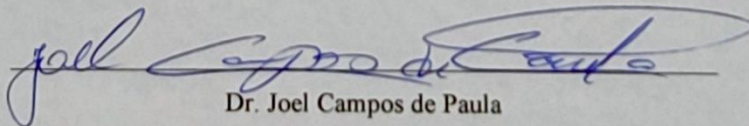
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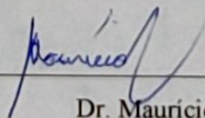
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Resumo

A hibridização interespecífica é um processo evolutivo significativo, capaz de influenciar a diversidade genética e o sucesso de invasões biológicas. Em *Cichla* (tucunarés), a hibridização é um fenômeno natural, que é intensificado pela ação humana, particularmente entre *Cichla piquiti* e *Cichla ocellaris* var. *kelberi*, duas espécies que coocorrem naturalmente na bacia Tocantins-Araguaia e foram amplamente introduzidas em outros sistemas. Esta tese investigou a dinâmica da hibridização e introgressão entre estas espécies através de uma abordagem integrativa, comparando populações em ambiente nativo (reservatório de Serra da Mesa) e não-nativo (reservatório de Três Marias). A estrutura do trabalho compreende uma revisão sistemática, análises genômicas via ddRAD-seq e morfometria geométrica. No primeiro capítulo, a revisão bibliográfica demonstrou que a hibridização em *Cichla* é geográfica e filogeneticamente disseminada em sua área nativa, conectando espécies distantes, mas geralmente em baixa frequência. Em contraste, em ambientes introduzidos, a hibridização é favorecida, resultando frequentemente em enxames híbridos onde a mistura genética é prevalente. No segundo capítulo, a análise de SNPs em escala genômica revelou padrões contrastantes de introgressão. Enquanto a população invasora em Três Marias apresentou extensa hibridização com retrocruzamentos avançados e alta heterozigosidade, a população nativa de Serra da Mesa, anteriormente considerada reprodutivamente isolada, revelou sinais de introgressão críptica. Além disso, análises de clinas genômicas na população invasora detectaram introgressão diferencial, identificando 46 loci com excesso de ancestralidade de *C. piquiti*, o que sugere a retenção seletiva de alelos específicos desta espécie apesar do fluxo gênico predominante em direção a *C. ocellaris* var. *kelberi*. Esses resultados indicam que as barreiras reprodutivas entre estas espécies são semipermeáveis e que o fluxo gênico, embora sutil no ambiente nativo, é exacerbado no contexto da invasão. Por fim, o terceiro capítulo integrou os dados genômicos à morfometria geométrica para avaliar as consequências fenotípicas da mistura. Foi detectada uma divergência ecomorfológica clara entre os parentais. Os híbridos exibiram morfologia estritamente intermediária, sem evidências de transgressão fenotípica. Houve uma forte correlação entre a forma do corpo e o índice híbrido

genômico, demonstrando que o fenótipo é um preditor confiável da ancestralidade genética nestas populações. Em conjunto, os resultados sugerem que a hibridização entre *C. piquiti* e *C. ocellaris* var. *kelberi* gera fenótipos viáveis e funcionais que, em ambientes alterados ou invadidos, podem contribuir para o sucesso ecológico e a persistência destas espécies.

Palavras-chave: ciclídeos; tucunaré; morfometria geométrica; espécies invasoras; ddRAD-seq.

Abstract

Interspecific hybridization is a significant evolutionary process capable of influencing genetic diversity and the success of biological invasions. In the genus *Cichla* (peacock bass), hybridization is a natural phenomenon that is intensified by human action, particularly between *Cichla piquiti* and *Cichla ocellaris* var. *kelberi*, two species that naturally co-occur in the Tocantins-Araguaia basin and have been widely introduced into other systems. This thesis investigated the dynamics of hybridization and introgression between these species through an integrative approach, comparing populations in a native environment (Serra da Mesa reservoir) and a non-native environment (Três Marias reservoir). The structure of this work comprises a systematic review, genomic analyses via ddRAD-seq, and geometric morphometrics. In the first chapter, the literature review demonstrated that hybridization in *Cichla* is geographically and phylogenetically widespread in its native range, connecting distant species, but generally at low frequency. In contrast, in non-native environments, hybridization is favored, frequently resulting in hybrid swarms where genetic admixture is prevalent. In the second chapter, genome-wide SNP analysis revealed contrasting patterns of introgression. While the invasive population in Três Marias presented extensive hybridization with advanced backcrossing and high heterozygosity, the native population of Serra da Mesa, previously considered reproductively isolated, revealed signs of cryptic introgression. Furthermore, genomic cline analyses in the invasive population detected differential introgression, identifying 46 loci with excess *C. piquiti* ancestry, suggesting selective retention of specific alleles from this species despite predominant gene flow toward *C. ocellaris* var. *kelberi*. These results indicate that reproductive barriers between these species are semi-permeable and that gene flow, although subtle in the native environment, is exacerbated in the invasion context. Finally, the third chapter integrated genomic data with geometric morphometrics to assess the phenotypic consequences of admixture. A clear ecomorphological divergence was detected between the parental species. Hybrids exhibited strictly intermediate morphology, with no evidence of phenotypic transgression. There was a strong correlation between body shape and the genomic hybrid index, demonstrating that phenotype is a reliable predictor of genetic ancestry in these populations. Taken together, the results suggest that hybridization between *C. piquiti* and *C. ocellaris* var.

kelberi generates viable and functional phenotypes that, in altered or non-native environments, may contribute to the ecological success and persistence of these species.

Keywords: cichlids; peacock bass; geometric morphometrics; invasive species; ddRAD-seq.

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Introdução geral

Os tucunarés (*Cichla*) constituem um importante grupo de ciclídeos neotropicais, tanto ecologicamente, como predadores de topo, quanto economicamente, como recurso de pesca de subsistência e esportiva (Winemiller et al. 2021). Sendo os maiores ciclídeos neotropicais, seu tamanho e comportamento agressivo os tornaram favoritos na pesca esportiva e recreativa. Essa popularidade levou à sua introdução em diversas regiões (Sastraprawira et al. 2020, Franco et al. 2021b), onde se tornaram invasores, impactando negativamente espécies nativas por meio da predação (Zaret & Paine 1973; Pelicice & Angelo 2009; Ota et al. 2019; Franco et al. 2021a). Duas das espécies de tucunaré mais introduzidas são o *Cichla ocellaris* Bloch & Schneider, 1801 e o *C. piquiti* Kullander & Ferreira, 2006, conhecidas popularmente como tucunaré amarelo e tucunaré azul, respectivamente. *Cichla piquiti* e *C. ocellaris* var. *kelberi* — uma unidade evolutivamente significativa previamente classificada como espécie — são totalmente simpátricas por toda extensão de suas áreas nativas, na bacia dos rios Tocantins-Araguaia (Kullander & Ferreira 2006, Willis et al. 2012). Essas espécies fazem parte de diferentes linhagens dentro da filogenia do gênero, sugerindo uma longa história de divergência evolutiva (Figura 1).

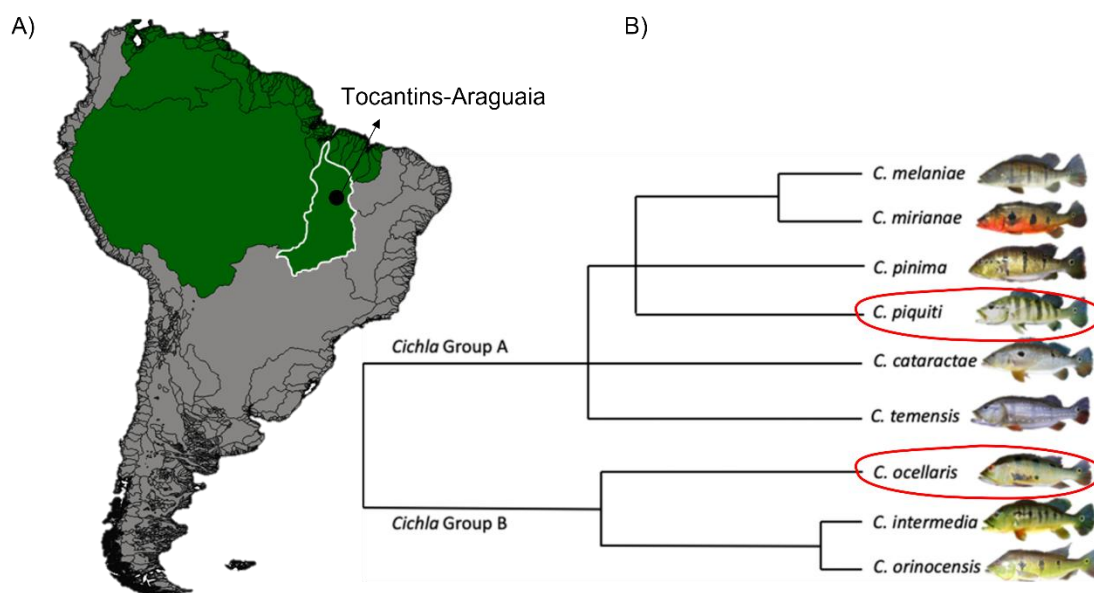


Figura 1. A) Mapa com distribuição nativa de espécies de *Cichla*, com destaque para a área nativa de *C. ocellaris* var. *kelberi* e *C. piquiti* (Bacia dos rios Tocantins-Araguaia).

B) Filogenia do gênero *Cichla* (Winemiller et al. 2021) com destaque para as espécies *C. ocellaris* e *C. piquiti*

Além da simpatria natural, *C. ocellaris* var. *kelberi* e *C. piquiti* coocorrem de forma introduzida em diversas localidades, principalmente no sul e sudeste do Brasil (Oliveira et al. 2006; Carvalho et al. 2014; Franco et al. 2021b; Nóbrega et al. 2023). Essa coocorrência, tanto em ambientes nativos quanto introduzidos, traz oportunidades de estudarmos mudanças nas interações entre essas espécies nesses diferentes contextos. Uma dessas interações, que tem consequências ecológicas e evolutivas, é a hibridização (Grabenstein & Taylor 2018). Na área nativa de *C. ocellaris* var. *kelberi* e *C. piquiti* a hibridização é considerada um fenômeno raro, com apenas um caso registrado até o momento (Carvalho et al. 2014). Em contraste, em ambientes onde ambas espécies foram introduzidas, a hibridização é frequente, de forma que híbridos compõem a maior parte das populações (Oliveira et al. 2006; Almeida-Ferreira et al. 2011; Nóbrega et al. 2023). Dessa forma, essas espécies são um bom modelo para estudarmos hibridização em populações nativas e introduzidas.

Nas populações nativas podemos, por exemplo, entender qual é o fluxo gênico natural entre essas espécies, e entender o papel evolutivo da hibridização para esse grupo. Entre os ciclídeos, as linhagens africanas estão entre os grupos de peixes de água doce mais estudados em relação à hibridização, com trabalhos demonstrando que esse fenômeno desempenhou um papel importante nas suas radiações adaptativas, especiação e evolução de novas características (Salzburger et al. 2002; Meier et al. 2017; Selz & Seehausen 2019). Em contraste, a hibridização entre os ciclídeos neotropicais tem sido negligenciada, apesar de serem um dos grupos mais diversificados de peixes de água doce na América do Sul e Central (Burress et al. 2015; Arbour et al. 2016).

Já em populações introduzidas, podemos entender os fatores relacionados à quebra de barreiras reprodutivas — considerando que essas espécies tendem a hibridizar mais frequentemente fora da sua área nativa — e entender como a hibridização influencia a invasão. No contexto de invasões biológicas, a hibridização entre espécies invasoras é preocupante devido ao potencial aumento da invasividade das populações envolvidas, o que pode agravar os impactos nos ecossistemas ocupados (Haynes et al. 2012). Por exemplo, o aumento da diversidade genética por meio da hibridização pode aliviar o efeito fundador. Além disso, a combinação de alelos previamente isolados pode gerar novos genótipos e aumentar os níveis de

heterozigosidade (Lee 2002; Zalapa et al. 2010). Um possível resultado dos maiores níveis de heterozigosidade é a heterose (vigor híbrido) (Birchler et al. 2006), que é o aumento de processos biológicos e comportamentais como crescimento, fecundidade, sobrevivência e agressividade, de indivíduos híbridos, quando comparados com os indivíduos parentais (Wohlfarth 1993; Rahman et al. 1995). Além disso, novos genótipos e fenótipos, assim como características intermediárias de híbridos, podem permitir que eles ocupem nichos antes não aproveitados pelas espécies parentais (Selz & Seehausen 2019). Esses fatores fazem com que a hibridização entre diferentes espécies de tucunares em ambientes não-nativos seja alarmante.

A identificação de hibridização ou introgressão pode ser um desafio quando os sinais morfológico e genômicos se diluem com retrocruzamentos sucessivos. Avanços nas tecnologias de sequenciamento permitem a análise de um grande número de polimorfismos de nucleotídeos únicos (SNPs), que superam significativamente os marcadores moleculares tradicionais (ex. microssatélites) em análises de hibridização (Camacho-Sanchez et al. 2020; Szatmári et al. 2021), além de fornecer melhor compreensão de padrões locos-específicos de introgressão (Ottenburghs et al. 2021). Nesse contexto, o protocolo de sequenciamento ddRAD-seq (“double digest restriction-site associated DNA sequencing”) destaca-se como uma das abordagens mais eficientes para a descoberta e genotipagem de SNPs em larga escala. Desenvolvida como uma evolução dos métodos de RAD-seq tradicionais, essa técnica utiliza simultaneamente duas enzimas de restrição para fragmentar o DNA genômico, permitindo uma redução precisa da complexidade do genoma ao focar apenas nos fragmentos flanqueados pelos sítios de corte de ambas as enzimas (Peterson et al. 2012). Essa técnica de representação reduzida do genoma não apenas diminui os custos de sequenciamento, viabilizando a análise de populações com grande número de indivíduos, mas também aumenta a reprodutibilidade dos dados entre amostras. Para estudos de hibridização, o ddRAD-seq é particularmente valioso pois gera milhares de marcadores distribuídos por todo o genoma, proporcionando a resolução fina necessária para distinguir populações e detectar introgressão mesmo em cenários de retrocruzamentos avançados. Paralelamente, abordagens de morfometria geométrica permitem quantificar com precisão diferenças morfológicas sutis entre indivíduos e detectar sinais fenotípicos de hibridização, complementando as análises genômicas (Kelly et al. 2025). Esses avanços possibilitam investigações mais abrangentes sobre as consequências genômicas da hibridização, incluindo a identificação de regiões genômicas que podem facilitar ou

resistir à introgressão. Essas informações são particularmente valiosas em espécies como *C. ocellaris* var. *kelberi* e *C. piquiti*, onde a hibridização pode influenciar traços adaptativos e o potencial invasivo.

Com isso, nesta tese, investigamos a hibridização e introgressão entre *C. ocellaris* var. *kelberi* e *C. piquiti* através de uma abordagem integrativa, utilizando dados genômicos (SNPs derivados de ddRAD-seq) e morfometria geométrica em dois cenários distintos. Comparamos o reservatório de Serra da Mesa (Goiás, 13° 58' 58.1376" S, 48° 21' 48.942" W), situado dentro da distribuição geográfica natural das espécies, onde estas coexistem historicamente sem registros de hibridização, com o reservatório de Três Marias (Minas Gerais, 18° 35' 57.3288" S, 45° 14' 10.6728" W), um ambiente de introdução mediada pela ação humana. Em Três Marias, *C. ocellaris* var. *kelberi* estabeleceu-se no início da década de 1980, seguida pela introdução de *C. piquiti* no final dos anos 2000 (Magalhães et al. 1996; Pinto-Coelho 2006; Carvalho 2009). Essa comparação nos permite testar diretamente como a dinâmica da hibridização difere entre um ambiente nativo e um não-nativo. Ao comparar os padrões de hibridização nesses ambientes distintos, visamos avaliar a extensão da hibridização tanto nas populações nativas quanto nas introduzidas, identificar regiões genômicas que evidenciem introgressão e explorar as potenciais consequências evolutivas e ecológicas do fluxo gênico entre essas espécies. Além disso, ao integrar a análise da forma corporal com um robusto arcabouço genético, buscamos compreender as consequências fenotípicas desse processo. Especificamente, avaliamos se a morfologia dos híbridos reflete suas proporções de ancestralidade genômica e se seus fenótipos são intermediários, similares aos parentais ou transgressivos. Dessa forma, este estudo visa elucidar não apenas os padrões genéticos da introgressão, mas também como ela molda a variação morfológica em populações de peixes invasores.

A presente tese encontra-se estruturada em três capítulos. O primeiro capítulo apresenta uma revisão sobre a hibridização envolvendo espécies de tucunaré (*Cichla*) em ambientes nativos e não nativos. O objetivo deste estudo é clarificar quais espécies hibridizam, detectar áreas geográficas potenciais para o estudo da hibridização e determinar sob quais condições ecológicas esse fenômeno tende a emergir, além de destacar as principais lacunas no conhecimento atual e delinear prioridades para pesquisas futuras. Na sequência, o segundo capítulo investiga a dinâmica genômica da introgressão entre *C. ocellaris* var. *kelberi* e *C. piquiti*, comparando populações nativas e introduzidas através de marcadores SNP via ddRAD-seq. Além disso,

sequenciaremos de um loco do DNA mitocondrial, que, por possuir herança matrilinear, pode fornecer informações acerca da direção de hibridização. Por fim, o terceiro capítulo integra a morfometria geométrica a esse arcabouço genético para avaliar as consequências fenotípicas da hibridização e a variação da forma corporal nos híbridos. Em conjunto, estes trabalhos buscam fornecer uma compreensão abrangente sobre o papel da hibridização na biologia evolutiva e na gestão de espécies invasoras.

Capítulo 1 — A review of hybridization between the Neotropical peacock basses *Cichla* (Perciformes: Cichlidae) in native and non-native environments.

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Keywords: neotropical cichlids; introgression; invasive species

Abstract

Hybridization is an important evolutionary process that can influence speciation, adaptation, and invasiveness. Within Neotropical cichlids, the genus *Cichla* (peacock bass) stands out for its ecological importance and frequent hybridization across both native and non-native ranges. Here, we present a comprehensive synthesis of published records of hybridization in *Cichla*. A systematic literature search in English, Portuguese, and Spanish identified 14 studies reporting 24 hybridization records involving six of the nine currently recognized species. Hybridization was documented across multiple major river basins within the genus native range (e.g., Orinoco, Amazon, Tocantins-

Araguaia) and has also been documented in invasive populations. In native environments, hybridization is geographically and phylogenetically widespread, linking distant lineages across multiple basins and suggesting that gene flow has played a role in the evolutionary history of *Cichla*. In non-native environments, hybridization has been documented primarily between *C. ocellaris* var. *kelberi* and *C. piquiti*, reflecting their widespread introduction. In these populations, hybrids often outnumber the parental species. These patterns suggest that both natural contact and anthropogenic factors such as dam construction, habitat alteration, and species introductions promote interspecific gene flow. Hybridization may facilitate introgression and adaptive evolution within *Cichla* natural range, but in non-native environments, it may also enhance invasiveness and ecological impact. We highlight the need for genomic analyses and ecological assessments to elucidate the drivers of reproductive barrier breakdown and determine the evolutionary and conservation implications of hybridization in Neotropical cichlids.

Introduction

Hybridization can be defined as the successful mating between individuals from two or more populations, that are distinguishable by heritable traits (Arnold 1997). Although it has been considered an evolutionary "dead end" in the past, it is now recognized as a key evolutionary process, influencing speciation and adaptation (Anderson 1953; Abbott et al. 2013). Hybridization is more frequent in fish than in other vertebrates, partly due to life history traits such as external fertilization and geographical overlap (Hubbs 1955; Campton 1987). The most recent comprehensive review of hybridization in freshwater fishes was conducted by Scribner et al. (2000), but it is now outdated due to significant advances in methods for detecting and analyzing hybrids. Targeted reviews have focused on hybridization within specific groups, such as catfishes (Adah et al. 2014; Gutiérrez-Barragán et al. 2025), sunfishes (Bolnick 2009; Shen & Wang 2018) and salmonids (Makhrov 2008). These group-specific syntheses are essential for identifying patterns that may be obscured in broader taxonomic surveys. Among cichlids, African lineages are among the most well-studied freshwater fish groups with respect to hybridization, with research showing that hybridization has played an important role in their adaptive radiations, speciation, and the evolution of novel traits (Salzburger et al. 2002; Meier et al. 2017; Selz & Seehausen

2019). In contrast, hybridization within Neotropical cichlids has been neglected, despite being one of the most diverse groups of freshwater fishes in South and Central America (Burress et al. 2015; Arbour et al. 2016).

Within Neotropical cichlids, the genus *Cichla* (peacock bass), stands out for its wide distribution and ecological importance (Kullander et al. 2006). Evidence indicates that hybridization in *Cichla* is both geographically and phylogenetically widespread (Willis et al. 2012), with multiple independent reports documenting hybrid individuals across their native range (Winemiller et al. 2021). In addition to ongoing hybridization, ancient introgression appears to have shaped the evolutionary history of the genus (Willis 2015; Willis et al. 2017). Over the past decades, *Cichla* species have also been widely introduced outside their native distribution to support sport fishing, increase fishery yields, or control other exotic species (Franco et al. 2022). In these non-native environments, their presence has often led to significant ecological impacts, including the decline of native fish assemblages (Zaret & Paine 1973; Pelicice et al. 2009; Menezes et al. 2012). In some of these regions, the introduction of multiple *Cichla* species has resulted in extensive hybridization, raising additional ecological concerns, including the potential for hybrid vigor and increased genetic variation, that may amplify ecological impacts and facilitate further expansion (Winemiller et al. 2021).

Despite a number of studies reporting hybrid individuals in *Cichla*, our understanding of the patterns and processes underlying these events remains fragmented. Early reports predated major taxonomic revisions of the genus, making it difficult to accurately determine which species were involved. Additionally, while hybridization has been documented in both native and non-native settings, the frequency and consequences appear to differ markedly between them. In this review, we synthesize published records of hybridization within *Cichla*, with the goal of clarifying which species hybridize, detect areas to focus hybridization studies on, and under which ecological conditions it tends to arise. Finally, we highlight key gaps in current knowledge and outline priorities for future research.

Methods

To compile records of hybridization involving *Cichla* species, we conducted a systematic literature search across multiple databases, including Google Scholar, Scopus, and ResearchGate. The search aimed to identify both peer-reviewed

publications and grey literature (e.g. theses) that detect or report evidence of hybridization. The primary search was performed using the keywords "peacock bass" OR "*Cichla*" AND "hybridization". To increase coverage, given that *Cichla* species are native to Brazil and other South American countries where much of the research is published in local languages, additional searches were conducted in Portuguese and Spanish. In Portuguese, we used the terms "tucunaré" OR "*Cichla*" AND "hibridização" OR "hibridação". In Spanish, we used: "pavón" OR "*Cichla*" AND "hibridación". Studies were included if they reported any evidence of hybridization involving *Cichla* species, regardless of whether it occurred in native or non-native environments. A hybridization record was defined as a link between the two peacock bass species interbreeding, the geographical coordinate of the system and the study reporting its occurrence. If a single study recorded more than one instance of hybridization, it was included in the database as a separate record. The aim was to gather information on various aspects of hybridization, including, but not limited to, the extent and frequency of hybridization events, factors influencing hybridization patterns, ecological and evolutionary implications of hybridization, and potential conservation implications for fish species involved. Furthermore, this review intends to provide insights into the methodological approaches employed to study hybridization in *Cichla*, highlighting the molecular techniques utilized. By consolidating the available knowledge on hybridization in this genus, this review contributes to a better understanding of the mechanisms and consequences of hybridization in *Cichla*.

In 2006, a taxonomic review conducted by Kullander and Ferreira, based on morphological and geographic data, as well as coloration patterns, identified a total of 15 distinct species of peacock bass. However, more recent research by Willis et al. (2012) delimited eight species on the basis of nuclear microsatellites and mitochondrial markers, suggesting that the remaining nominal species represent regional stocks within broader taxa. These findings have been supported by subsequent molecular studies (Willis et al. 2013; Willis et al. 2014; Willis et al. 2017). Additionally, a new species has been described recently (Sabaj et al. 2020). Hence, in this review, we adopted the species delimitation by Willis et al. (2012), while following Winemiller et al. (2021) denomination, and recognized the following groups as valid: *C. ocellaris* sensu lato (as per Willis et al. 2012), *C. cataractae* Sabaj Pérez, López-Fernández, Willis, Hemraj, Taphorn & Winemiller, 2020, *C. intermedia* Machado-Allison, 1971, *C. melaniae* Kullander & Ferreira, 2006, *C. mirianae* Kullander & Ferreira, 2006, *C. orinocensis*

Humboldt, 1821, *C. pinima* sensu lato (as per Willis et al. 2012), *C. piquiti* Kullander & Ferreira, 2006, *C. temensis* Humboldt, 1821. Furthermore, we also considered hybridization between the different lineages of *C. ocellaris* sensu lato, namely *C. ocellaris* var. *ocellaris*, *C. ocellaris* var. *monoculus*, *C. ocellaris* var. *kelberi* and *C. ocellaris* var. *pleiozona*. We also recognize the diversity within *C. pinima* sensu lato (Willis et al., 2012; Willis 2017), as to better interpret hybridization patterns.

Data extracted from the selected articles included the species hybridizing, geographical locations with coordinates, type of environment (native or non-native) and hybrid detection methods. Furthermore, the contributing factors to hybridization were also examined, encompassing ecological factors (e.g. habitat overlap) and anthropogenic factors (e.g. species introductions, habitat modifications). The gathered information served to provide a comprehensive understanding of the patterns, locations, and underlying factors influencing hybridization among peacock bass species.

Results

Search results

Our systematic search across three languages yielded a total of 421 references. After removing duplicates and screening for relevance, 14 studies met the criteria for inclusion in this review. Although the number of included studies is relatively low, they encompass a wide geographic range across the natural and introduced distribution of *Cichla*, and report hybridization involving six of the nine currently recognized species. This highlights the relevance of the available data and reinforces the value of synthesizing these records to better understand the patterns, contexts, and drivers of hybridization in the genus.

Species involved in hybridization

Some of the studies reporting hybridization between *Cichla* species were conducted prior to the taxonomic revision of the genus by Kullander & Ferreira (2006). As a result, the species names used in those earlier reports may differ from the nomenclature currently recognized. In this review, we retain the species names as originally reported in each study, and later discuss how those identifications might be interpreted in light of current knowledge on the taxonomy and distribution of *Cichla*. The only exception is the hybridization record by Oliveira et al. (2006), which reported

hybridization between *C. cf. monoculus* cf and *C. sp. 'Azul'* in the Paraná Basin, as later work confirmed these as *C. ocellaris* var. *kelberi* and *C. piquiti* (Almeida-Ferreira et al. 2011).

A total of 24 recent hybridization records were identified, involving 13 nominal forms (Table 1), these are: *C. cf. monoculus*, *C. monoculus*, *C. sp. 'Azul'*, *C. intermedia*, *C. kelberi*, *C. nigromaculata*, *C. ocellaris*, *C. ocellaris* var. *kelberi*, *C. orinocensis*, *C. pinima*, *C. piquiti*, *C. temensis*, *C. vazzoleri*. Most studies were able to identify both parental species involved in hybridization, with the exception of Quadros et al. (2020), which identified *C. vazzoleri* as one parental species but could not distinguish between *C. monoculus* or *C. temensis* as the other, due to identical patterns in 5S rDNA position in chromosomes for these two species. Hybridization was detected both between closely related species, such as *C. intermedia* × *C. orinocensis* and *C. pinima* × *C. piquiti*, and between more genetically distant species, such as *C. piquiti* × *C. ocellaris* var. *kelberi* and *C. temensis* × *C. monoculus*. The majority of the hybridization records (17 out of 24) involved species from the different major clades within *Cichla* (Group A and Group B, Figure 1 and Figure S1). Out of the six hybridization records outside of peacock bass native range, five involved *C. ocellaris* var. *kelberi* × *C. piquiti*, while one reported the intraspecific hybridization between *C. ocellaris* var. *monoculus* × *C. ocellaris* var. *kelberi*.

Table 1. Hybridization between *Cichla* species reported in the literature.

Species pair	Location	Status	Reference
<i>C. monoculus</i> × <i>C. temensis</i>	Tocantins river (Tucuruí reservoir)	Native	Andrade et al. (2001)
<i>C. monoculus</i> × <i>C. temensis</i>	Tocantins river (Estreito)	Native	Andrade et al. (2001)
<i>C. monoculus</i> × <i>C. temensis</i>	Tapajós River (Santarém)	Native	Andrade et al. (2001)
<i>C. monoculus</i> × <i>C. temensis</i>	Uatumã River (Balbina reservoir)	Native	Andrade et al. (2001)

<i>C. monoculus</i> × <i>C. temensis</i>	Uatumã River (Balbina reservoir)	Native	Brinn et al. (2004)
<i>C. monoculus</i> × <i>C. temensis</i>	Uatumã River (downstream of Balbina reservoir)	Native	Teixeira & Oliveira (2005)
<i>C. monoculus</i> × <i>C. temensis</i>	Lago Catalão (confluence of Negro and Solimões Rivers)	Native	Teixeira & Oliveira (2005)
<i>C. cf. monoculus</i> × <i>C. sp. 'Azul'</i>	Paraná River Basin (Itaipu Reservoir and floodplain)	Invasive	Oliveira et al. (2006)
<i>C. intermedia</i> × <i>C. monoculus</i>	Mavaca River	Native	Willis et al. (2007)
<i>C. kelberi</i> × <i>C. piquiti</i>	Upper Paraná River Basin (Itaipu reservoir)	Invasive	Almeida-Ferreira et al. (2011)
<i>C. kelberi</i> × <i>C. piquiti</i>	Upper Paraná River Basin (UHE Eng. Sérgio Motta)	Invasive	Almeida-Ferreira et al. (2011)
<i>C. orinocensis</i> × <i>C. intermedia</i>	Parguaza River	Native	Willis et al. (2012)
<i>C. orinocensis</i> × <i>C. monoculus</i>	Negro River (Novo Airão)	Native	Willis et al. (2012); Willis et al. (2014)
<i>C. orinocensis</i> × <i>C. monoculus</i>	Preto da Eva River	Native	Willis et al. (2012); Willis et al. (2014)

<i>C. nigromaculata</i> × <i>C. temensis</i>	Mavaca River	Native	Willis et al. (2012)
<i>C. pinima</i> × <i>C. piquiti</i>	Tocantins River (downstream of Tucuruí reservoir)	Native	Willis et al. (2012); Willis et al. (2017)
<i>C. ocellaris</i> × <i>C. temensis</i>	Cuyuni River	Native	Willis et al. (2012); Willis et al. (2015)
<i>C. orinocensis</i> × <i>C. temensis</i>	Guri reservoir	Native	Willis et al. (2012)
<i>C. kelberi</i> × <i>C. piquiti</i>	Tocantins river (Tucuruí reservoir)	Native	Carvalho et al. (2014)
<i>C. vazzoleri</i> × <i>C. monoculus</i> or <i>C. temensis</i>	Uatamã River (Balbina reservoir)	Native	Quadros et al. (2020)
<i>C. kelberi</i> × <i>C. monoculus</i>	São João River Basin (Juturnaíba reservoir)	Invasive	Diamante et al. (2020)
<i>C. ocellaris</i> var. <i>kelberi</i> × <i>C. piquiti</i>	Paraíba do Sul River Basin (Lajes reservoir)	Invasive	Nóbrega et al. (2023)
<i>C. ocellaris</i> var. <i>kelberi</i> × <i>C. piquiti</i>	São Francisco River Basin (Três Marias reservoir)	Invasive	Nóbrega et al. (in prep.)
<i>C. ocellaris</i> var. <i>kelberi</i> × <i>C. piquiti</i>	Tocantins River (Serra da Mesa reservoir)	Native	Nóbrega et al. (in prep.)

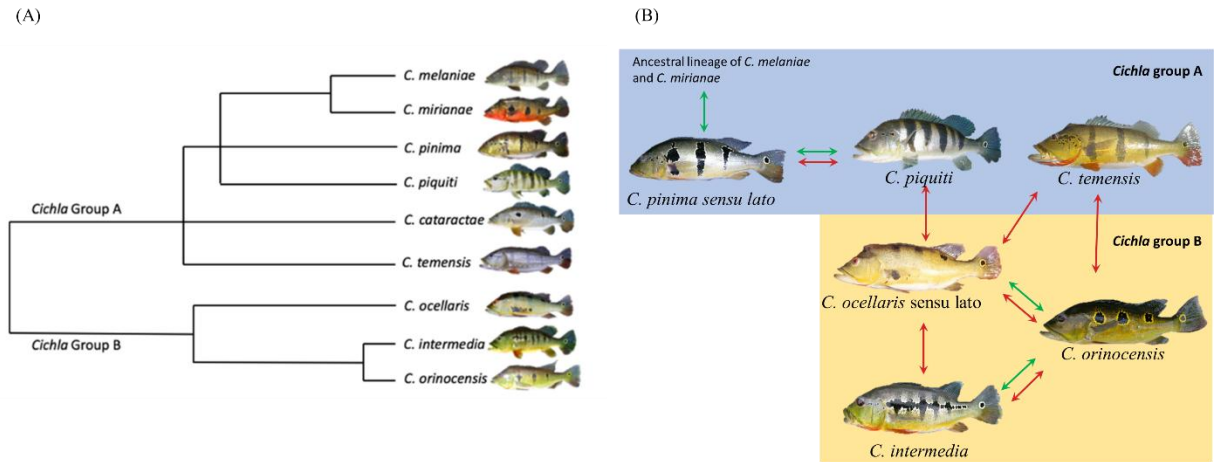


Figure 1. *Cichla* phylogeny (from Winemiller et al. 2021) (A), species with records of hybridization (B). Red arrows indicate recent hybridization and green arrows indicate ancient introgression. Colored rectangles indicate species group within genus phylogeny.

Hybridization localities

Hybridization has been documented in both native and non-native environments (Figure 2). In native ranges, hybridization was recorded mainly in the Orinoco Basin, in the interface or overlap of the distribution of *C. intermedia*, *C. orinocensis*, *C. temensis* and *C. ocellaris* var. *ocellaris*, in the Middle Amazon, where *C. pinima*, *C. orinocensis*, *C. temensis* and *C. ocellaris* var. *monoculus* are recorded, and in the Lower Tocantins, where *C. ocellaris* var. *kelberi* and *C. piquiti* sympatric range marginally overlaps with *C. pinima*. In the invaded range, hybridization records remain restricted to South and Southeastern Brazil, in the Paraná, Paraíba do Sul, and São João river basins.

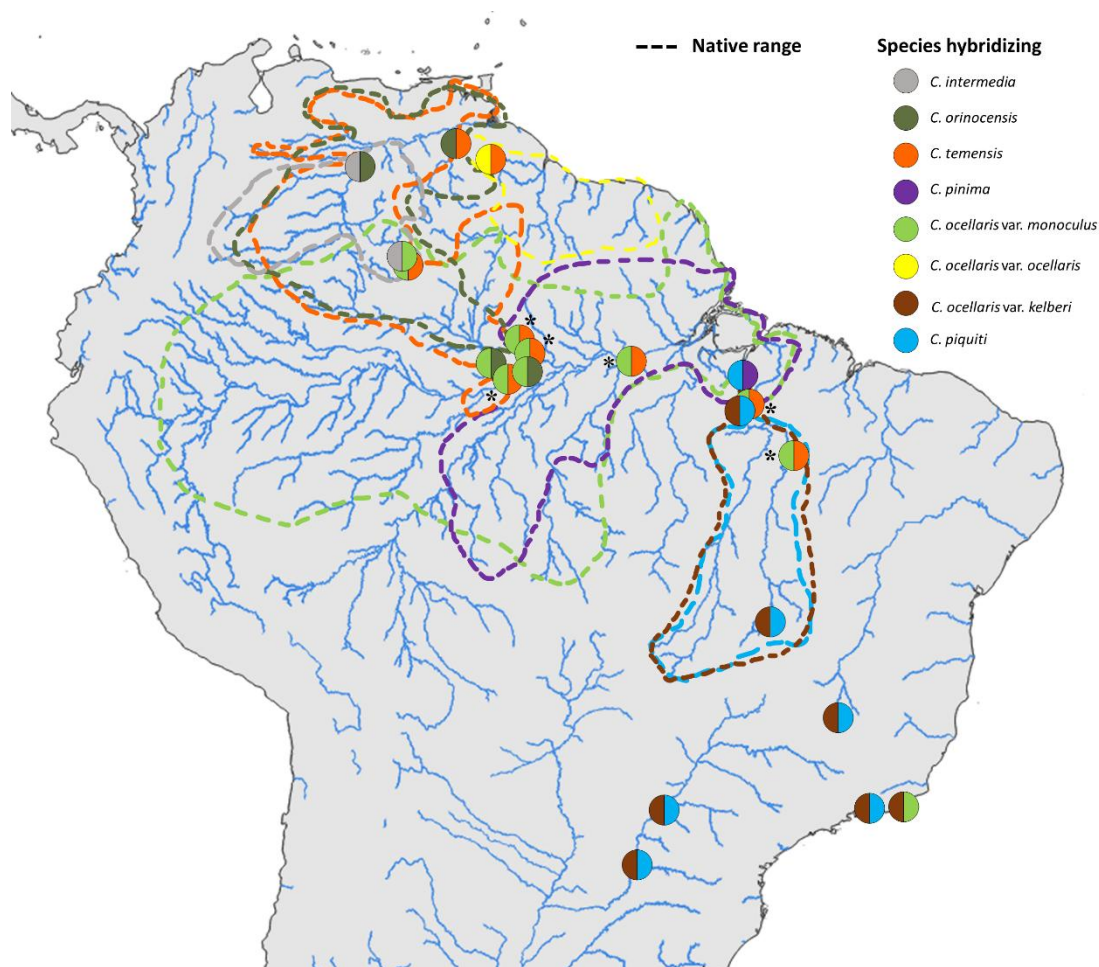


Figure 2. Map showing locations of recorded hybridization events among *Cichla* species. Native ranges are outlined with dashed lines, with each species' range indicated by its corresponding color. South American major rivers are shown (Strahler order ≥ 6 ; HydroRIVERS v1.0). Pie charts indicate the species identified as hybridizing in each record, according to the original studies. These identifications reflect the taxonomy used at the time and may differ from current classifications (see Discussion). Pie charts marked with an asterisk correspond to records published prior to the taxonomic revision by Kullander & Ferreira (2006).

Factors influencing hybridization

Although no study has directly tested the factors associated with hybridization between peacock bass species, several conditions known to promote hybridization in fishes are present in the areas where these events were recorded. Among the 14 individual locations with hybridization records in native environments, seven are situated in regions heavily impacted by human activities, most notably areas affected by dam construction, either within reservoirs or in downstream stretches where river

flow has been altered (e.g. Guri, Balbina, Tucuruí reservoirs). The remaining seven records were found in relatively undisturbed habitats, indicating that hybridization in *Cichla* is not limited to human-altered systems. In non-native environments, five of the six individual locations with hybridization records are artificial reservoirs. The sole exception was the Upper Paraná River floodplain, downstream of the Itaipu hydroelectric dam.

Methods of hybrid identification

A variety of methods have been used to detect hybridization between *Cichla* species (Figure 3). These include protein electrophoretic patterns, cytogenetic analyses, and karyological studies (Brinn et al., 2004; Teixeira & Oliveira, 2005; Quadros et al., 2020). Mitochondrial DNA (mtDNA) markers, are also widely used (Andrade et al. 2001; Willis et al. 2012; Nóbrega et al. 2022). Nuclear DNA (nDNA) markers, such as Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR), were introduced to complement mtDNA data (Oliveira et al., 2006; Almeida-Ferreira et al. 2011; Diamante et al. 2020). Since the last decade, microsatellite markers were also widely applied in the detection of hybridization between *Cichla* species (Willis et al. 2012; Carvalho et al., 2014; Willis et al. 2015; Willis et al. 2017; Nóbrega et al., 2023). More recently, double-digest restriction-site associated DNA sequencing (ddRAD-seq) has been used to detect hybridization in both native and non-native environments (Nóbrega in prep.).

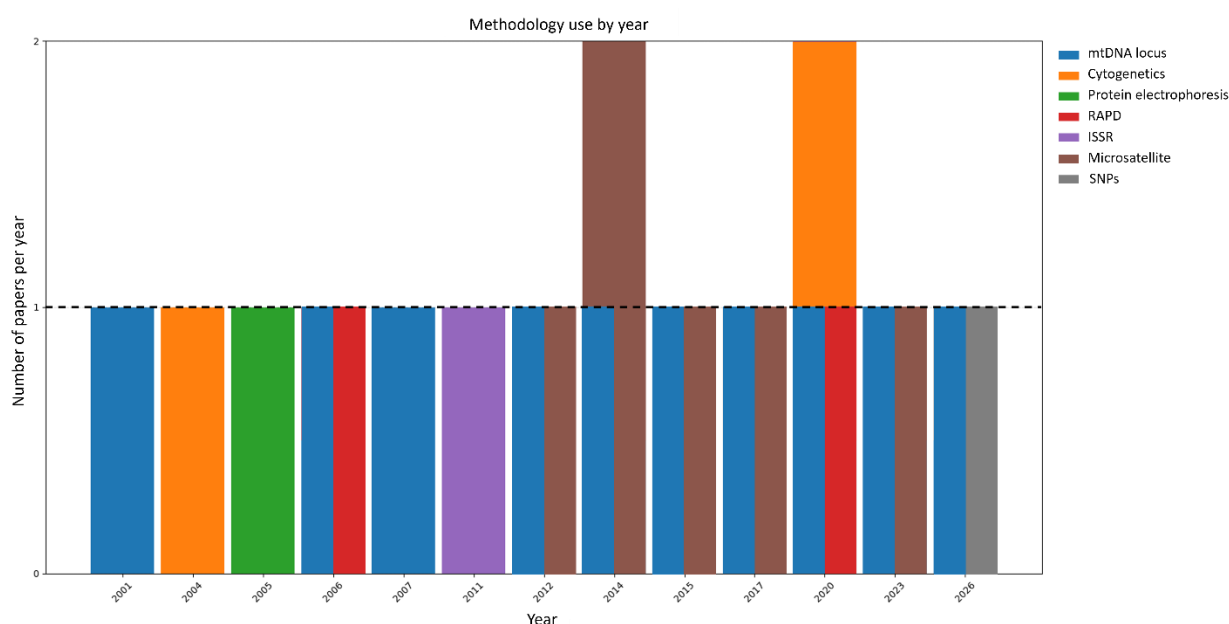


Figure 3. Methods used to detect hybridization between *Cichla* species in published papers. Papers that used more than one methodology are represented by multicolored bars. Legend: RAPD - Random Amplified Polymorphic DNA; ISSR - Inter Simple Sequence Repeat.

Discussion

Reinterpreting early hybridization records

Reinterpreting the 24 hybridization records identified in this review under the current taxonomic framework shows that the 13 nominal forms correspond to hybridization involving at least six of the nine currently recognized *Cichla* species: *C. intermedia*, *C. ocellaris* sensu lato (includes *C. ocellaris*, *C. monoculus*, *C. nigromaculata*, *C. kelberi*, *C. ocellaris* var. *kelberi*), *C. orinocensis*, *C. pinima* (includes *C. pinima*, *C. vazzoleri*), *C. piquiti*, and *C. temensis*. However, given that several of the original reports used informal or provisional names (e.g., *C. cf. monoculus*, *C. sp. "Azul"*), these identifications should be viewed as interpretative rather than definitive. This is particularly important because some of the earliest studies (e.g., Andrade et al. 2001; Brinn et al. 2004; Teixeira & Oliveira 2005) were published prior to the major revision by Kullander & Ferreira (2006), and by the molecular analyses of Willis et al. (2012). These earlier studies did not generate or publish DNA sequence data, which prevents direct comparisons with current molecular datasets.

For instance, records from the Tocantins (Tucuruí, Pará; Estreito, Maranhão) and Tapajós (Santarém, Pará) rivers identified hybridization between "*C. temensis*" and "*C. monoculus*". However, based on current distributions, the Tocantins basin is inhabited by *C. pinima*, *C. ocellaris* var. *kelberi*, and *C. piquiti*, while the Lower Tapajós hosts *C. pinima* and *C. ocellaris* var. *monoculus* populations. The Balbina reservoir (Uatumã River, Amazonas) presents a similar taxonomic conflict. Andrade et al. (2001), Brinn et al. (2004) and Teixeira & Oliveira (2005) reported hybridization between "*C. monoculus*" and "*C. temensis*" in the Balbina reservoir, which is located at the center of *C. ocellaris* var. *monoculus* distribution, and at the edge of *C. pinima* and *C. temensis* distribution. The most comprehensive studies on the geographic distribution of *Cichla* indicates that *C. pinima* is restricted to between the Middle and Upper course of the Uatumã River, and *C. temensis* to the Lower Uatumã (Kullander & Ferreira 2006; Willis et al. 2012; Willis et al. 2017). Personal observations by LNS,

who has conducted fieldwork in the Balbina reservoir, reported only *C. pinima* and *C. ocellaris* var. *monoculus*. However, Winemiller et al. (2021) mentions that *C. temensis* was stocked in Balbina reservoir, although no reference is provided. Furthermore, Quadros et al. (2020) and Escobar et al. (2024) analyzed samples reportedly of *C. temensis* from Balbina reservoir, although these studies did not use any diagnostic markers to confirm fish identity, only classifying individuals based on coloration. *Cichla pinima* and *C. temensis* can appear very similar to each other, making their identification based on coloration difficult, especially when fish are juveniles, where both species express the “paca” colour pattern.

Therefore, recognizing the limitations at the time of these earlier studies, the reports of hybridization between “*C. temensis*” and “*C. monoculus*” in the Tocantins River, Tapajós River and Balbina reservoir should be interpreted with caution and warrant re-evaluation using updated taxonomic frameworks and molecular tools. Nonetheless, the occurrence of hybridization in these locations remains well supported, with uncertainty relating to which species were actually involved. Regarding the hybridization records within Balbina reservoir, we suggest that targeted surveys and molecular analyses are needed to confirm which *Cichla* species occur in this reservoir.

Hybridization in native environments

In native environments, hybridization tends to occur in certain contact zones. Notable examples include the Lower Tocantins River, where the sympatric distribution of *C. ocellaris* var. *kelberi* and *C. piquiti* marginally overlaps with the range of *C. pinima*; the Middle Amazon, where *C. temensis*, *C. pinima*, *C. ocellaris* var. *monoculus*, and *C. orinocensis* occur either sympatrically or parapatrically; and the Orinoco Basin, which harbors overlapping distributions of *C. intermedia*, *C. orinocensis* and *C. temensis*. Even though hybridization in native areas is geographically and phylogenetically widespread, the frequency of detected hybrids within populations remains relatively low. In Willis et al. (2012), only ~2% (26 out of 1,177) of the sampled specimens were identified as hybrids based on mismatch between morphology and mtDNA haplotype. In Carvalho et al. (2014) ~5.2% (2 out of 38) of the sampled specimens in Tucuruí were identified as hybrids based on microsatellite data. A similarly low number of hybrids were detected in other studies within *Cichla* native range (Andrade et al. 2001; Teixeira & Oliveira 2005; Quadros et al. 2020).

It is worth noting that in many of the areas where hybridization was detected, the total number of specimens analyzed was relatively small (Willis et al. 2012; Carvalho et al. 2014; Nóbrega et al. in prep). Even in Willis et al. (2012), which remains the most comprehensive survey of hybridization across *Cichla* native range, sampling per locality was often limited. This raises the possibility that hybridization may be more widespread than reported, particularly at low frequencies that are difficult to detect without extensive sampling. Still, even at low frequencies, hybrids facilitate the transfer of alleles between species through introgression, which may have important evolutionary consequences (Mallet et al. 2005). This phenomenon is observed on a small scale through SNP-based analyses (Nóbrega et al. in prep.). Although only three individuals were identified as recent hybrids between *C. ocellaris* var. *kelberi* and *C. piquiti* in Serra da Mesa reservoir (Tocantins), most sampled specimens showed some signal of introgression due to backcrossing. Other studies that employed high-resolution markers, such as microsatellites, also detected late-generation hybrids and backcrosses involving different species pairs, suggesting that introgression is common (Willis et al. 2012; Carvalho et al. 2014).

When examining the species and localities with confirmed hybridization events, an interesting pattern emerges: hybridization forms a potential chain that spans the entire native range of the genus (Figure 4). At the northern edge of *Cichla* distribution, in the Orinoco basin, *C. intermedia* hybridizes with *C. orinocensis*, which in turn both hybridize with *C. temensis*. *Cichla temensis* hybridizes with *C. ocellaris* var. *monoculus* around the connection of the Orinoco with the Negro River, and in the Middle Amazon River, which may hybridize with *C. pinima* in the Tapajós River. *Cichla pinima* hybridizes with *C. piquiti* in the Lower Tocantins, which ultimately hybridizes with *C. ocellaris* var. *kelberi* at the southeastern limit of the genus range. Such pattern, if confirmed by genomic analyses of shared ancestry, could facilitate the long-distance spread of alleles across the genus, similar to observations in other interconnected systems like the loach genus *Triplophysa* in the Tibetan Plateau (Qian et al. 2023). This widespread introgression may be particularly significant for the diversification and adaptation of *Cichla*, due to its slow rate of molecular evolution (Farias et al. 1999; López-Fernández et al. 2004).

However, it is important to recognize that hybridization does not always lead to introgression. Another way of interpreting this pattern is that *Cichla* species tend to form hybrid zones at the interface of their geographic distributions. This is more evident

between *C. intermedia* × *C. temensis* in Mavaca River, *C. temensis* × *C. ocellaris* var. *ocellaris* in Cuyuni river, in the Middle Amazon where multiple species occur, and between *C. piquiti* and *C. pinima* the Lower Tocantins. In such cases, if hybrids exhibit reduced fitness, natural selection may favor traits that prevent interspecific mating, a process known as reinforcement (Noor 1999). This may be supported by the low frequency of hybrids in natural areas. Rather than facilitating gene flow, hybridization in these zones could therefore strengthen reproductive barriers, maintaining or even enhancing species boundaries over time.

Importantly, reinforcement and introgression are not mutually exclusive. Genomic studies increasingly show that species boundaries are semipermeable, allowing the exchange of neutral or adaptive alleles while maintaining reproductive isolation at loci under divergent selection, reflecting differential introgression rates across the genome (Payseur 2010; Harrison & Larson 2016). Future studies should prioritize regions where hybridization has already been reported to determine whether these sites represent true hybrid zones. If confirmed, such zones could be used to investigate the spatial and temporal dynamics of hybridization and offer valuable insights into the evolutionary processes shaping the diversification of *Cichla* (Hewitt 1988).

Analyses by Willis (2015) and Willis et al. (2017) have already shown that introgression has influenced the evolutionary history of *Cichla*. Incongruences between mitochondrial and nuclear phylogenies suggested historical gene flow between *C. orinocensis* and a lineage ancestral to *C. ocellaris* var. *monoculus* (Willis et al. 2012; Willis et al. 2014). Similar patterns were observed in *C. pinima* populations, which appear to have experienced past introgression with *C. piquiti* and an ancestral lineage of *C. mirianae* and *C. melaniae* (Willis et al. 2012; Willis 2017). Given the extent of hybridization observed between *Cichla* species, it is likely that additional ancient introgression events have occurred within the genus. However, the absence of detectable mtDNA signals in earlier work, combined with the lack of genomic studies, has hindered our ability to identify them. Future studies incorporating higher-resolution genomic approaches, such as single nucleotide polymorphisms (SNPs) or whole-genome sequencing (WGS), could provide a more comprehensive picture of historical and contemporary gene flow in *Cichla*. These methods would allow to detect subtle signatures of introgression, distinguish between ancient and recent hybridization events,

and assess the genomic regions most affected by gene flow, improving our understanding of how gene flow has affected the evolutionary history of *Cichla* species.

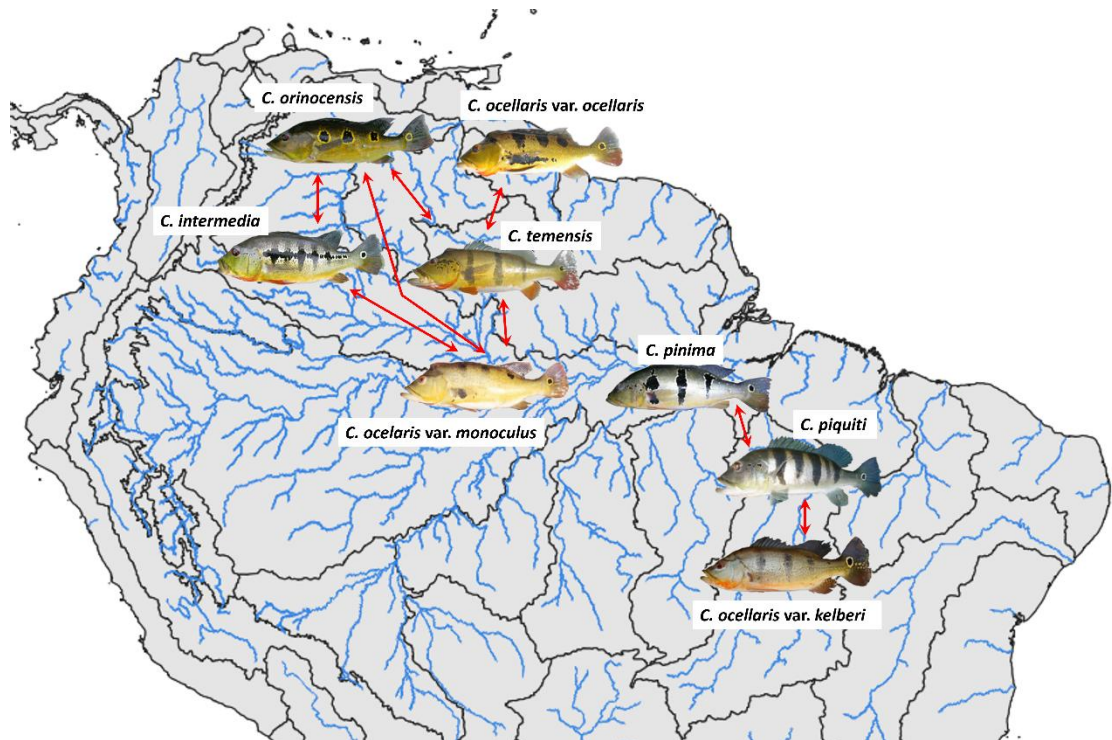


Figure 4. Hybridization relationships (red arrows) across *Cichla* native range considering published records. Note that hybridization between *C. ocellaris* var. *monoculus* and *C. pinima* is also likely in Santarém (see discussion).

Hybridization in invasive populations

In contrast to native areas, hybridization in non-native environments appears to result in a high proportion of hybrids within populations. However, it is important to note that current results are limited by the taxonomic bias of available records. Among the six documented cases of hybridization in non-native environments, five involve *C. ocellaris* var. *kelberi* and *C. piquiti*, while one involves a case of intraspecific hybridization between *C. ocellaris* var. *kelberi* and *C. ocellaris* var. *monoculus*. This likely reflects the fact that these are the most introduced *Cichla* species (Franco et al. 2022). As a result, patterns observed in introduced populations largely reflect interactions between this particular pair of species. Hybrids between *C. ocellaris* var. *kelberi* and *C. piquiti* in invaded areas often make up the majority of sampled populations. For instance, in the Upper Paraná River floodplain, 52 out of 68 analyzed specimens exhibited fixed nuclear DNA fragments from both species, indicating advanced stages of hybridization (Oliveira et al. 2006). Similarly, in Lajes Reservoir

(southeastern Brazil), 18 out of 28 individuals were identified as hybrids based on nuclear microsatellite data (Nóbrega et al. 2023). At Três Marias Reservoir, SNP-based analyses revealed that 19 out of 24 sampled individuals were recent hybrids (Nóbrega et al. in prep.).

When comparing the number of invasive *Cichla* populations where hybridization has been reported, to the full extent of their introduced range worldwide, it becomes evident that hybridization is still underdetected. Multiple regions, particularly in South America, but also in parts of North America and Asia, harbor two or more introduced peacock bass species (Franco et al. 2022). Yet, documented cases of hybridization remain restricted to populations in South and Southeastern Brazil. This is likely not due to the absence of hybridization, but rather a lack of molecular studies targeting invasive *Cichla* populations. Anecdotal evidence, including photographs and videos available online from anglers, often reveals individuals with intermediate phenotypes that do not match the diagnostic features of any known species, suggesting the presence of hybrids. Similarly, hybrid specimens are frequently observed in the aquarium trade. Therefore, expanding molecular monitoring of invasive *Cichla* populations across their introduced range is essential.

This lack of studies is particularly concerning given the potential ecological consequences of hybridization in invasive populations. Invasive peacock bass are known to affect native communities directly and indirectly, causing declines in species richness and abundance, as well as disrupting ecosystem processes (Zaret & Paine 1973; Pelicice et al. 2009; Menezes et al. 2012; Franco et al. 2022). Therefore, hybridization among introduced *Cichla* species may further enhance the invasiveness of these populations, increasing their ecological impact (Haynes et al. 2012). By introducing alleles previously absent in a given species, this process can boost genetic diversity, mitigate founder effects, and generate novel genotypes that may facilitate adaptation to new environments (Lee 2002; Zalapa et al. 2010).

Anthropogenic drivers of hybridization in Cichla

The specific factors driving hybridization between *Cichla* species remain unclear. However, the elevated frequency of hybrids in non-native environments may provide clues about the breakdown of reproductive barriers (Viard et al. 2020). Hybridization between introduced species is generally associated with secondary contact between lineages that were previously allopatric (Ellstrand et al. 2000;

Ottenburghs 2021), as seen with *C. ocellaris* var. *monoculus* and *C. ocellaris* var. *kelberi* introduced to the Juturnaíba Reservoir. However, this do not explain hybridization between naturally sympatric species like *C. ocellaris* var. *kelberi* and *C. piquiti*. Therefore, other factors must be driving hybridization between them in non-native environments. Notably, five of the six records of *Cichla kelberi* × *Cichla piquiti* hybridization in non-native environments come from artificial reservoirs, which are widely recognized as drivers of hybridization due to habitat alteration and ecological homogenization (Agostinho et al. 2007). Altered environmental cues such as water temperature and flow patterns might reduce the effectiveness of prezygotic barriers, facilitating interspecific mating (Chunco et al. 2014; Rosmetsch et al. 2020). However, in reservoirs located within these species native range, hybrids are still relatively rare (Willis et al. 2012; Carvalho et al. 2014).

The breakdown of prezygotic barriers may be linked to altered reproductive phenology. *Cichla* populations in undisturbed environments exhibits pronounced seasonality in reproductive activity (Jepsen et al., 1999; Lowe-McConnell, 1969; Muñoz et al., 2006; Santos et al., 2011; Winemiller, 2001). In native, but altered, environments (e.g. reservoirs in native areas), such as Lajeado reservoir, *C. piquiti* displays an extended breeding season with a peak from February to September (Marto et al. 2015), whereas reproductive females of *C. ocellaris* var. *kelberi* are observed throughout a smaller period, primarily between October and December (Gaspar et al. 2004). Another study also detected that *C. ocellaris* var. *kelberi* and *C. piquiti* in Lajeado differ markedly in their reproductive periods (Andrade & Pelicice 2022). In Balbina reservoir, *C. pinima* (vazzoleri) spawned throughout the year, but mainly between October and January. In constrast, several studies have reported that *Cichla* introduced populations remain reproductively active throughout the year (Chellapa et al., 2003; Gomiero et al., 2009; Vieira et al. 2009; Normando et al. 2009; Guedes et al. 2020).

If damming alters reproductive patterns in both native and non-native areas, yet hybridization remains much less common in native reservoirs, then additional factors unique to non-native areas must also be driving higher hybridization rates. One possible explanation is an imbalance in the abundance of each species at the time of introduction. According to the "Hubbs' principle" or "desperation hypothesis," individuals may hybridize when conspecific mates are rare (Hubbs 1955). This situation often arises in introduced systems, where population sizes can be small and uneven during the early

stages of establishment. In every non-native site where hybridization between *C. ocellaris* var. *kelberi* and *C. piquiti* has been reported, *C. ocellaris* var. *kelberi* was introduced first and became established before *C. piquiti* arrived. The introduction of *C. piquiti*, likely in smaller numbers, may have led to limited conspecific mating opportunities, promoting interspecific hybridization with the already abundant *C. ocellaris* var. *kelberi*.

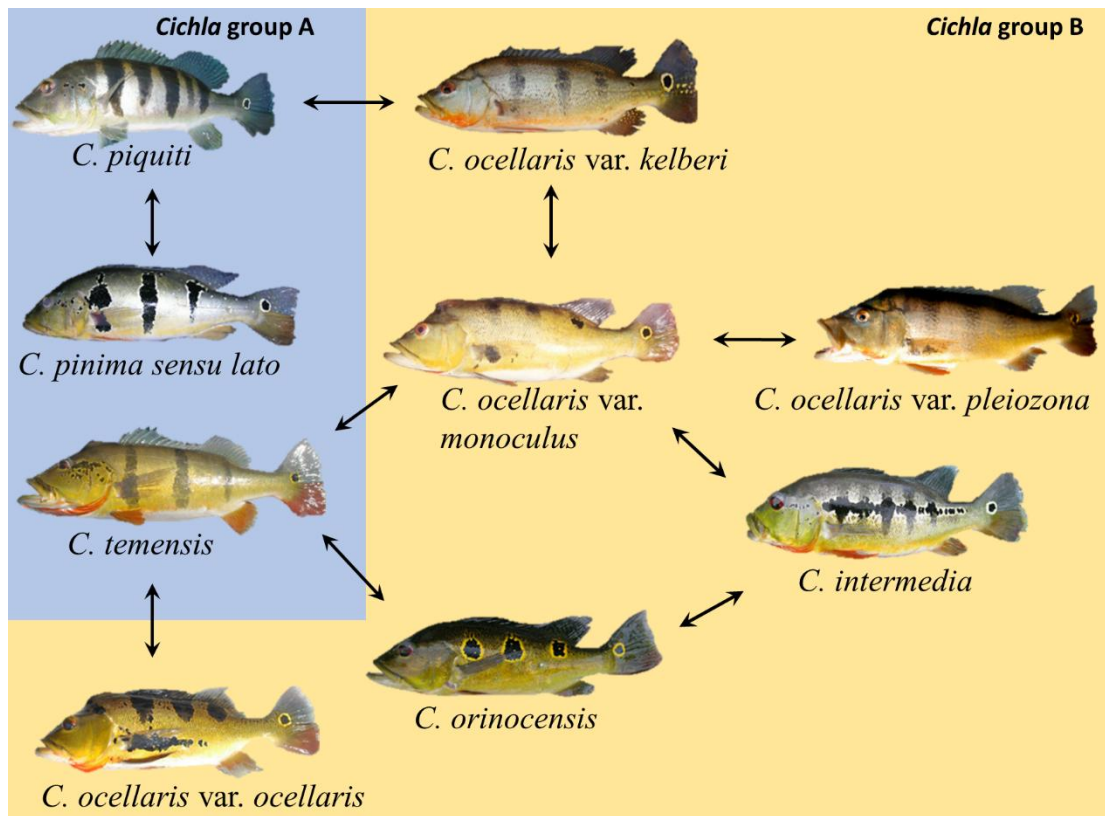
Future directions

This review highlights both the growing body of evidence for hybridization within *Cichla* and the many unresolved questions that remain. In native environments, increasing sample sizes in previously identified hybridization hotspots, along with the application of genomic tools (e.g. RAD-seq, whole-genome sequencing), could reveal whether stable hybrid zones are present, providing important opportunities to investigate evolutionary processes in *Cichla*. Such data can clarify whether hybridization results in widespread gene flow, localized introgression of adaptive alleles, or reinforcement of reproductive barriers. Expanded molecular screening of invasive populations is also needed, but it must be accompanied by efforts to quantify the ecological consequences of hybridization. Hybrid individuals may express novel phenotypes, greater ecological plasticity, or enhanced fitness, potentially amplifying their impact on native communities. Clarifying whether hybridization contributes to increased invasiveness or altered trophic interactions is essential for understanding risks and guiding management strategies. Finally, experimental approaches, such as controlled crosses and common garden experiments, can be used to test mechanisms such as mate choice and hybrid fitness.

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Supplementary Figures



Supplementary Figure 1. Species pairing (records of hybridization) considering each *C. ocellaris sensu lato* ESU separately. Colored rectangles indicate species group within genus phylogeny.

Capítulo 2 — Evidence of hybridization and introgression between two fully sympatric peacock bass (*Cichla piquiti* and *C. ocellaris* var. *kelberi*) from ddRAD-seq data: subtle in native, conspicuous in invasive populations

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Abstract

Hybridization and introgression are recognized as important drivers of evolutionary dynamics and invasive success in freshwater fishes. The sympatric occurrence of closely related species in both native and non-native environments provides a natural experiment to examine hybridization dynamics in these different contexts. *Cichla ocellaris* var. *kelberi* and *C. piquiti* are sympatric congeners that are frequently introduced to the same areas. Here, we investigated the genomic patterns of hybridization between *Cichla ocellaris* var. *kelberi* and *C. piquiti* across native (Serra da Mesa, SM) and invasive (Três Marias, TM) reservoirs using genome-wide ddRAD-seq and mtDNA control region sequencing. All mtDNA haplotypes were species-specific, with some shared between the native and introduced populations. SNP-based analyses of population admixture revealed clear species separation in both areas and gave clear signs of hybridization and bidirectional backcrossing in the invasive population. The D_3 tests detected cryptic introgression in the native reservoir.

Heterozygosity was the highest in admixed individuals and in *C. ocellaris* var. *kelberi*, suggesting possible asymmetric introgression. Bayesian genomic cline analyses of hybrids from the invasive population identified 106 α outlier loci, with deviations detected in both directions, indicating genomic heterogeneity in introgression. Although both sampling sites are affected by damming, hybridization was far more frequent in the invasive populations, possibly facilitated by extended reproductive periods and imbalances in species abundance. Our results illustrate how the permeability of reproductive barriers depends on ecological context and highlight the value of genome-wide analyses for uncovering complex patterns of hybridization and introgression.

Introduction

Once deemed rare and an “evolutionary dead end”, hybridization has been increasingly recognized as a widespread phenomenon with significant implications (Taylor & Larson 2019). By transferring genetic material between species, introgressive hybridization can influence adaptation, drive speciation and invasiveness (Ellstrand & Schierenbeck 2000; Peñalba et al. 2024). This process can lead to both negative and positive outcomes. Whereas it may result in the loss of locally adapted traits or the homogenization of gene pools, it can also introduce novel genetic variation that improves adaptation and survival in new or changing environments (Arnold 1997; Ellstrand & Schierenbeck 2000; Abbott et al. 2013; Barraclough 2024; Peñalba et al. 2024). In addition to natural hybridization, anthropogenic activities are driving an increase in hybridization among taxa that rarely or do not interbreed under undisturbed conditions. This is primarily associated with human-assisted translocations, habitat modifications, and climate change (Crispo et al., 2011; Vallejo-Marín and Hiscock, 2016; Grabenstein and Taylor, 2018; Ottenburghs, 2021). One possible consequence is hybridization between two invasive species, which may amplify their invasiveness or ecological impact by combining adaptive traits (Chouvenc et al. 2015; Coulter et al. 2019; Nóbrega et al. 2023). Although this scenario is relatively rare compared to hybridization events between invasive and native taxa, it occurs relatively frequently in peacock basses.

Peacock basses (*Cichla*) are an important group of Neotropical cichlids, both ecologically, as top predators, and economically, as a popular angling resource

(Winemiller et al. 2021). As the largest of the Neotropical cichlids, they are favored for recreational and sport fishing due to their size and aggressive behavior. This popularity has led to their introduction into numerous regions (Sastraprawira et al. 2020), where they become invasive, negatively impacting native species (Zaret and Paine 1973; Pelicice and Angelo 2009; Ota et al. 2019; Franco et al. 2021a). Often, two or more *Cichla* species are introduced to the same area, creating many opportunities for anthropogenic hybridization (Franco et al. 2021b). These ecological characteristics and introduction patterns make *Cichla* a compelling model for investigating the evolutionary and ecological consequences of hybridization in invasive species. Among *Cichla* species, *C. ocellaris* and *C. piquiti* are the most frequently introduced, often co-occurring in non-native environments (Franco et al. 2022). Their natural sympatry across their entire native range makes them an interesting case of naturally co-occurring species that re-encounter each other in non-native environments. These species belong to each of the two major clades within *Cichla*, suggesting a long history of independent evolution, with divergence time estimates ranging from ~7 Ma based on mitochondrial clocks to ~17 Ma in multilocus time-calibrated analyses (Willis et al. 2012; Willis et al. 2013). Despite their long independent evolutionary history, these species retain broadly similar ecological niches, as both are large piscivorous predators with comparable reproductive behaviors, although differing in body size, coloration patterns, and microhabitat use (Kullander et al. 2006 Winemiller et al. 2021).

Extensive evidence shows hybridization and introgression between *C. ocellaris* var. *kelberi* and *C. piquiti* in non-native environments (Oliveira et al., 2006; Almeida-Ferreira et al., 2011; Nóbrega et al., 2023). In contrast, reports of hybridization in their sympatric native range are limited to two putative hybrids in Tucuruí reservoir, which were detected through the analysis of eight microsatellite loci (Carvalho et al. 2014). This low frequency of hybrids in native environments, even in altered habitats, has also been observed across other *Cichla* species in broad geographic surveys using microsatellite analysis (Willis et al. 2012). Advances in sequencing technologies now enable the analysis of a large number of SNP markers, which significantly outperform traditional molecular markers (e.g. microsatellites) in admixture analyses (Camacho-Sanchez et al. 2020; Szatmári et al. 2021), as well as provide a better understanding of loci-specific patterns of introgression (Ottenburghs et al. 2021). These advancements have enabled more comprehensive investigations into the genomic consequences of hybridization, including the identification of genomic regions that may facilitate or

resist introgression. Such insights are particularly valuable for species such as *C. ocellaris* and *C. piquiti*, where hybridization may influence adaptive traits and invasive potential.

In this study, we investigate hybridization and introgression between *C. ocellaris* var. *kelberi* and *C. piquiti* using genome-wide SNP data from individuals sampled in two reservoirs. Serra da Mesa represents a reservoir within the native range, where these species have historically coexisted with no records of hybridization. In contrast, the Três Marias reservoir is located outside the natural distribution of these species and represents a site of human-mediated introduction. In Três Marias, *C. ocellaris* var. *kelberi* was first introduced and became established in the early 1980s, followed by the introduction of *C. piquiti* in late the 2000s (Magalhães et al. 1996; Pinto-Coelho 2006; Carvalho 2009). By comparing these two reservoirs, we can directly test how hybridization and introgression differ between a native environment and a non-native environment where both species were introduced.

We hypothesize that, despite significant environmental alteration due to damming in both regions, (1) hybridization is found in low frequency in the native-range population, whereas introgression is pervasive in the introduced population. We also hypothesize that (2) despite potential founder effects, the introduced population maintains or even exceeds the genetic diversity of the native population due to introgression. Additionally, given the earlier introduction and longer establishment of *C. ocellaris* in Três Marias, as well as potential differences in abundance, we predict that (3) introgression is asymmetric, with genomic material from *C. piquiti* (the later introduction) more frequently incorporated into *C. ocellaris* var. *kelberi*. By comparing patterns of genomic admixture across these distinct environments, we aim to assess the extent of hybridization in both native and non-native populations, identify genomic regions exhibiting evidence of introgression, and explore the potential evolutionary and ecological consequences of gene flow between these species. Our findings, based on the first genome-wide assessment of introgression between *Cichla* species, provide insights into the role of hybridization in shaping genetic variation, particularly in the context of invasive species.

Materials and methods

Sampling and Study Areas

Specimens of *Cichla piquiti* and *Cichla ocellaris* var. *kelberi* were collected from Serra da Mesa reservoir (13° 58' 58.1376" S, 48° 21' 48.942" W), where both species are native and occur in sympatry, and from Três Marias reservoir (18° 35' 57.3288" S, 45° 14' 10.6728" W), where both species were introduced. Serra da Mesa reservoir (SM) was built in 1998 in the Tocantins-Araguaia River basin.

Three peacock bass species are known to occur in the Tocantins-Araguaia River basin, *Cichla pinima*, *C. piquiti* and *Cichla ocellaris* var. *kelberi*. While *C. pinima*'s distribution is restricted to the downstream region of the basin and *C. piquiti* inhabits mostly the upstream region, *C. ocellaris* var. *kelberi* can be found across its full extent, occurring in sympatry with the other two (Willis et al. 2012). Being located near the headwaters of the Tocantins-Araguaia River basin, only *C. piquiti* and *C. ocellaris* var. *kelberi* are present in SM. Três Marias reservoir (TM) is located in the São Francisco River basin, outside of *Cichla*'s natural distribution. *Cichla ocellaris* var. *kelberi* was introduced to TM in the early 1980s (Magalhães et al. 1996; Carvalho 2009), while the introduction of *C. piquiti* likely occurred in the late 2000s, as the species was not recorded in surveys of the ichthyofauna of the Três Marias reservoir that occurred between 2001 and 2005 (Pinto-Coelho 2006). A total of 48 fish were collected by angling with artificial baits, comprising 24 specimens from each reservoir.

Morphological identification

Kullander & Ferreira (2006) identified 15 species of *Cichla* based exclusively on morphological data and coloration patterns. However, more recent genetic analyses have suggested that only nine species can be clearly delimited, with several of these being identified as polytypic species, reflecting intraspecific diversity (Willis et al., 2012; Willis et al., 2013, Sabaj et al. 2020; Winemiller et al., 2021). Here, we used the morphological characterization established by Kullander & Ferreira (2006) to identify *Cichla* specimens, but adopted the species delimitation from Willis et al. (2012), which classifies *C. kelberi* as a regional stock or variant of *C. ocellaris*. In SM, 12 specimens were morphologically identified as *C. ocellaris* var. *kelberi* and 12 as *C. piquiti*, while in TM, 10 were identified as *C. ocellaris* var. *kelberi*, six as *C. piquiti* and eight as putative hybrids.

Total DNA extraction and mtDNA sequencing

Total genomic DNA extractions were performed from 25 mg of fin tissue using the commercial kit NucleoSpin Tissue (Macherey–Nagel GmbH and Co. KG), according to the manufacturer's instructions. The quality and integrity of the extracted DNA were assessed by electrophoresis on a 1% agarose gel in 0.5X TAE buffer using a Lambda PstI ladder, followed by spectrophotometric analysis with a Nanodrop and quantification with a Qubit fluorometer.

To assess mtDNA diversity, identify species-specific haplotypes, and infer the maternal direction of introgression, we sequenced the hypervariable mtDNA Control Region (CR). The CR was amplified using the pair of primers tPro2-5 (5'-ACCC TAA CTC CCA AAG C-3') and HN-20-3 (5'-GTG TTA TGC TTT AGT TAA GC-3') (Lee et al. 1995; Palumbi 1996). PCR reactions for CR amplification occurred with a final volume of 25 µL containing 1 unit (U) of Taq DNA polymerase (ThermoScientific Inc., USA), 1 × PCR buffer with NH₄SO₄, 2.5 mM MgCl₂, 0.16 mM dNTPs (ThermoScientific Inc., USA), 8 pmol of each primer and 5 ng of genomic DNA. The PCR conditions occurred with an initial step of 5 min at 94 °C, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. To confirm amplification, an aliquot of the final PCR products was stained with GelRedR and applied in a 1% agarose gel in 0.5X TAE buffer. The amplicons were purified and sequenced by Macrogen Inc (Seoul, South Korea) via Sanger sequencing by capillary electrophoresis using the same PCR primers. Because CR in *Cichla* has a poly-T region, sequencing of the 3' portion is problematic (Willis et al. 2007). Therefore, each sample was sequenced in duplicate using only the tPro2-5 primer. Sequences are available in GenBank under accession numbers XXXXX-XXXXX.

mtDNA sequences processing and analysis

The Control Region sequences were manually edited in MEGA11 (Tamura et al. 2021) and aligned with other *Cichla* sequences taken from GenBank by the ClustalW algorithm. A maximum likelihood phylogenetic tree was constructed using MEGA11. The best-fitting substitution model was selected using the "Find Best DNA/Protein Models" function, which identified the Tamura-Nei model with gamma-distributed rate variation and a proportion of invariant sites (TN93 + G + I) as the most appropriate. Tree reconstruction was performed under this model, with branch support estimated using 1,000 bootstrap replicates.

ddRAD-seq library preparation and sequencing

Library preparation and sequencing were performed by Floragenex (USA, www.floragenex.com). Genomic DNA was digested with the restriction endonucleases PstI and MseI and processed into a RAD library similar to the method of Truong et al. (2012). Briefly, ~125 ng of genomic DNA was digested for 120 min at 37°C in a 20 µL reaction with 2.5 units (U) of PstI and 2.5 U of MseI (New England Biolabs [NEB]). After digestion, samples were heat-inactivated for 10 min at 80°C, followed by the addition of 2.5 µL of 1 µM PstI P1 adapter, and 0.1 µL of 250 µM MseI P2 adapter. PstI P1 adapters each contain a unique multiplex sequence index (barcode), which is read during the first five nucleotides of the Illumina sequence read. P1 and P2 adapters were added to each sample along with 100 U T4 DNA Ligase (high concentration [HC], NEB), 0.250 U MseI, and 0.260 U PstI for a final reaction volume of 25 µL which was then incubated at 37°C for 180 minutes. Samples were diluted 1:10 in water, and 2.5 µL of this product was used in a PCR amplification with 10x PCR Buffer 1 (Applied Biosystems), dNTP Mix (IDT; 0.1 µl at 20 nM concentration), 6.01 µL H₂O, 15 ng MseI primer, 2.5 ng PstI primer, and 0.2 U AmpliTaq DNA Polymerase (Applied Biosystems). The PCR program is as follows: a initial heating step of 2 min 72°C, 13 cycles of 30 sec 94°C, 2 min 67°C, decreasing -0.7°C per cycle, 2 min 72°C, followed by 37 cycles of 30 sec 94°C, 2 min 58°C, 2 min 72°C. Genomic libraries were sequenced using a single lane of an SP flow cell on the Illumina NovaSeq 6000 platform, generating 1×118 bp single-end reads.

Raw reads quality filtering and SNP calling

Initially, the quality of each sample was assessed using FastQC (Andrews et al. 2010). We used the Stacks v. 2.68 pipeline (Rochette et al. 2019) for demultiplexing and SNP discovery. Raw reads were demultiplexed and low-quality reads were filtered using the ‘process_radtags’ program. Reads were considered low-quality if their average quality score, calculated using a sliding window of 15% of the read length, dropped below a Phred score of 10 (equivalent to a 90% probability of being correct). Reads with errors in the barcode or the RAD cut site exceeding the allowed threshold were also discarded. Due to the limited availability of high-quality reference genomes for species closely related to peacock bass, loci were assembled both with and without (*de novo*) a reference genome. Therefore, some results will be presented for the analysis of loci assembled through both methods. For the *de novo* loci discovery, we used

Stack's 'denovo_map.pl' program, which executes each of Stack's components individually. First, the 'ustacks' program builds loci in each sample by aligning reads into exactly-matching stacks, then, the 'cstacks' program creates a catalog of consensus loci from multiple samples by merging stacks that match across samples, and finally the 'sstacks' program matches each individual's stacks against the catalog. As the assembly of loci *de novo* is affected by multiple parameters of these programs, we followed the R80 optimization protocol outlined in Rochette & Catchen (2017) and Rivera-Colón & Catchen (2022). This optimization process looks for the assembly parameters (-M, -m and -n) that maximize the number of R80 loci in the dataset (polymorphic loci present in at least 80% of the samples). As per these protocols' recommendations, we tested multiple values of -M/-n (M=n), keeping -m as the default value of 3. The optimal assembly parameter value for our dataset was an -M/-n of 4. The reference-based loci assembly was performed with the program 'ref_map.pl' with default parameters, after alignment of each sample's reads with the reference genome of *Oreochromis niloticus* (GenBank GCA_001858045.3) After generating the *de novo* and reference-based catalogues of loci, the final SNPs datasets were generated through Stacks 'populations' program. We kept SNPs present in loci found in at least 80% of all individuals (-r 0.8), with a Minimum Allele Frequency higher than 0.02 (--min-maf 0.02). For analysis affected by linkage disequilibrium, the --write-single-snp parameter was employed, restricting to one SNP per locus in order to minimize linkage between SNPs.

Population structure and admixture analysis

To assess population structure and visualize genetic differentiation we first performed Principal Component Analysis (PCA) on genetic distance matrices using the function *cmdscale* in the R software. The genetic distance matrices were calculated from dataset1_ref and dataset1_denovo in Plink v1.9 (Purcell et al. 2007), which uses the IBS (identity-by-state) measure. We conducted PCA analyses on the full dataset, including all specimens, and separately by location. The first two principal components (PC1 and PC2) were plotted in R using the *ggplot* function. We also analyzed ancestry relationships among our samples using the programs ADMIXTURE v1.3 (Alexander et al. 2009) and fastStructure v1.0 (Raj et al. 2014). As ADMIXTURE and fastStructure assume that loci are independent, both analyses were performed using dataset2_ref and dataset2_denovo, which are restricted to one SNP per locus. In ADMIXTURE, the

optimal number of groups (K) was determined by performing the cross-validation procedure, outlined in the user manual, for which we applied a 10-fold cross-validation. In fastStructure, optimal K was identified through the chooseK.py tool. To test if remaining LD was still affecting the results, we LD pruned dataset2_ref, removing SNPs with pairwise $r^2 > 0.5$ in blocks of 50 SNPs with a 10 SNPs moving window using ‘--indep-pairwise 50 10 0.5’ in Plink, and repeated both analyses.

To better understand the power of our loci panel to detect late generation hybrids in ADMIXTURE and fastStructure, we followed the approach described by Lavretsky et al. (2016). Using the R script *R.code.4.hybrid.Simulations.r* (Dryad: doi:10.5061/dryad.g3g65) we simulated first-generation hybrids (F1) and successive backcross generations (F2–F10) into either *C. piquiti* or *C. ocellaris* var. *kelberi* parental populations. Briefly, we generated ten F1 hybrids by randomly sampling alleles from the parental gene pools at bi-allelic SNP positions, with allele selection weighted by their respective frequencies. Five of these F1 hybrids were then backcrossed to either parental population for nine generations to model genomic composition across hybrid classes. For the parental gene pool, we selected five specimens of each species with posterior probabilities >0.99 in ADMIXTURE and highest average genetic distance to the other parental species. To further explore patterns of genomic co-ancestry among individuals, we also conducted an analysis with the R package fineRADstructure (Malinsky et al. 2018). fineRADstructure uses a Markov chain Monte Carlo (MCMC) clustering algorithm to infer fine-scale population structure by analyzing co-ancestry patterns among genotypes derived from RADseq data. It is particularly effective for detecting subtle population subdivisions and genetic relationships within and between closely related groups. fineRADstructure has no limitations regarding the analysis of linked loci, in fact, it benefits from LD between multiple polymorphisms within the same RAD locus (Malinsky et al. 2018), therefore, we used dataset1_ref on this analysis.

Admixed ancestry and genetic diversity

To evaluate the relationship between genetic admixture and genetic diversity, we plotted individuals ADMIXTURE ancestry fractions (dataset2_ref, K = 2) against their observed heterozygosity. The hybrid index was defined as the proportion of *C. piquiti* ancestry for each specimen. Individuals with an ancestry fraction below 0.01 or above 0.99 were classified as parental, while individuals with intermediate values were

classified as hybrids. Observed heterozygosity was estimated in the Stacks ‘populations’ program.

Test for introgression

To further investigate the possibility of introgression in the native area, we used the D_3 test (Hahn & Hibbins, 2019), which is related to the ABBA-BABA test. However, instead of inferring introgression based on the frequencies of alternative gene tree topologies (ABBA-BABA), the D_3 is based on the expectation of symmetry in branch lengths given incomplete lineage sorting, which eliminates the need for an outgroup. Therefore, it serves as an alternative to the ABBA-BABA test when an appropriate outgroup is unavailable for the species or populations being analyzed. The D_3 test follows a tree structure in the format ((A, B) C), where A and B represent closely related populations or individuals, and C is the potential introgressor. The D_3 is calculated by the formula $D_3 = \frac{dB-C-dA-C}{dB-C+dA-C}$. We applied the D_3 test considering both sample A and sample B from the same population (SM) to avoid potential bias, as the alternative population (TM) exhibited extensive introgression. While this approach may reduce sensitivity to introgression, any positive signal observed under these conditions would be more significant. Genetic distance was calculated as the percentage of differing sites across an alignment of 27,034 10-kb non-continuous blocks. To assess significance, the D_3 was computed using 1000 block bootstraps with replacement, followed by a Z-test to determine whether it differed significantly from zero. When D_3 is negative and significant, it indicates introgression between samples B-C, while a positive and significant value suggests introgression between samples A-C. To investigate the potential introgression of *Cichla piquiti* alleles into *C. ocellaris* var. *kelberi*, we tested all possible combinations of A and B using samples from *C. ocellaris* var. *kelberi*, while C was represented by samples of *C. piquiti*. Similarly, to detect introgression of *C. ocellaris* var. *kelberi* alleles into *C. piquiti*, we reversed the sample positions, with A and B as samples from *C. piquiti* and C as a sample of *C. ocellaris* var. *kelberi*. We fixed each sample at a time as A, while rotating the B and C samples. This resulted in 132 combinations of ((A, B) C) for each sample.

Outlier loci and patterns of introgression

To assess differential introgression (loci-specific patterns of introgression), we used the *bgc* software (Gompert & Buerkle 2012) through the *ClineHelpR* package (Martin et al. 2021). *bgc* is based on the Bayesian genomic cline model (Gompert & Buerkle 2011), which estimates locus-specific patterns of introgression by modeling allele frequency shifts across hybrid zones. This approach allows for the identification of loci with significantly higher or lower introgression than expected under neutral conditions, highlighting candidate regions that may be under selection or exhibit unique introgression dynamics. Due to the small number of hybrids in the native reservoir, and the small number of parental specimens in the invaded area, we applied the genomic cline analysis with hybrids from TM populations, using the SM pure samples as reference populations. As the genetic differences between *C. ocellaris* var. *kelberi* from SM and TM, highlighted by the clustering analyses (see results), could introduce bias, we ran the genomic cline analysis with a filtered SNP dataset to reduce the effects of population structure. The filtered dataset was generated from *dataset2_ref*, filtering loci with $F_{ST} > 0.05$ between all *C. ocellaris* var. *kelberi* from SM and the eight individuals from TM with the highest ancestry proportion of *C. ocellaris* var. *kelberi* based on the ADMIXTURE analysis. We assessed the effectiveness of this filtering by examining whether it reduced the genetic differentiation and structure between them in a PCA (Figure S7). Then, we ran the *ClineHelpR* package on the filtered dataset to analyze loci-specific patterns of introgression in the invasive populations with minimized population structure effects. We ran three independent MCMC chains of 50,000 steps each, discarding the first 10,000 steps as burn-in. Samples were taken from the posterior distribution every 10 steps. Loci were considered outliers when the 95% credible interval of α or β did not overlap zero ($qn = 0.95$).

Gene Ontology enrichment analysis

We conducted gene ontology (GO) enrichment analysis of the candidate genes identified through *bgc*. Using the *biomaRt* (Durinck et al., 2009) package in R, we retrieved gene annotations from the *O. niloticus* reference genome to obtain gene names and descriptions. We then performed GO enrichment analysis using *topGO* (Alexa & Rahnenführer, 2018), which accounts for the hierarchical structure of GO terms to reduce biases associated with highly annotated genes. A *nodeSize* threshold of 10 was used to exclude GO terms associated with fewer than 10 annotated genes. The *weight01*

algorithm was applied, which downweights broad GO terms to improve specificity, and Fisher's exact test was used to identify significantly enriched biological processes, molecular functions, and cellular components among our candidate genes.

Results

Sequencing of mtDNA

We obtained Control Region (CR) sequences for all 48 samples, consisting of 494 nt after alignment. Species-specific haplotypes were identified by alignment with CR sequences of all other *Cichla* species available in Genbank. All haplotypes identified in both areas belong to either *C. ocellaris* var. *kelberi* or *C. piquiti*. In SM, a single haplotype of *C. piquiti* and two of *C. ocellaris* var. *kelberi* were identified, while in TM a single haplotype from each species was detected. The haplotypes found in TM are also present in SM, corresponding to the shared *C. piquiti* haplotype and one of the two *C. ocellaris* var. *kelberi* haplotypes (Figure S1).

Referenced and de novo loci discovery and SNP selection

Approximately 458M reads were sequenced from the 48 samples. After Stack's 'process_radtags' program, 20.5% of total reads were discarded due to the barcode not being found, less than 0.1% were discarded due to low-quality, and 4.2% were discarded as the RAD cutsite could not be found. Approximately 345M reads were retained, amounting to 75.4% of the total reads. Retained reads per sample varied between 4,240,522 and 8,931,497, while the percentage of reads retained per sample varied between 87.6% and 98.5%. After filtering with the 'populations' program, four separate SNPs datasets were created, two generated from the reference-based loci discovery, and two generated from the denovo loci discovery. Each SNP dataset, with its generating parameters and number of SNPs, are listed in Supplementary Table 1.

Population structure and admixture analysis

The principal components analyses with all samples based on both dataset1 (ref and denovo) were highly similar (Figure 1A and 1B). The first two principal components separated individuals into three main groups: *C. piquiti* from both reservoirs, with minor separation between SM and TM samples along PC2, *C. ocellaris*

var. *kelberi* from SM, and *C. ocellaris* var. *kelberi* from TM. The first principal component (PC1) explained 86.81% and 87.85% of the variation in the reference and de novo datasets, respectively, and clearly distinguished *C. piquiti* from *C. ocellaris* var. *kelberi* across both localities. The second principal component (PC2), which explained 4.14% and 3.96% of the variation, captured differences between populations of the same species across reservoirs, particularly among *C. ocellaris* var. *kelberi* individuals. In both reservoirs, *C. ocellaris* var. *kelberi* displayed greater intraespecific variation based on the lack of clustering along PC2. Morphologically identified putative hybrids from the invasive populations were positioned between the clusters of the parental species, or closer to *C. ocellaris* var. *kelberi*, suggesting that backcrosses with *C. ocellaris* var. *kelberi* are more common.

ADMIXTURE's cross-validation errors of $K = 2$ and $K = 3$ were highly similar (Figure S2). In fastStructure, the optimal K was identified by the chooseK.py tool as $K = 3$. When setting $K = 2$ in both analyses, clusters are representative of *C. piquiti* and *C. ocellaris* var. *kelberi* from both sampling areas (Figure 2 and Figure S3). When setting $K = 3$, *C. ocellaris* var. *kelberi* from the different sampling sites are also differentiated, suggesting different ancestry. While hybridization based on both ADMIXTURE's and fastStructure's ancestry fractions was very evident in the invasive populations, signals of admixture in the native populations were less pronounced. In the native populations, using a threshold of < 0.99 to identify hybrids, ADMIXTURE analyses (based on dataset2_ref and dataset2_denovo) detected 1-4 individuals with admixed ancestry at $K = 2$ and $K = 3$. In contrast, fastStructure did not identify any admixed individuals using the same threshold. In the invasive populations, ADMIXTURE detected 15- 19 individuals with admixed ancestry, while fastStructure identified 12-17 individuals. A single individual (TM3) from the invaded reservoir exhibited incongruence between its mtDNA haplotype and nuclear ancestry. Despite this specimen having ADMIXTURE and fastStructure q -values > 0.99 , indicating a strong genetic assignment to *C. piquiti*, its mtDNA control region haplotype corresponded to *C. ocellaris* var. *kelberi*.

fineRADstructure coancestry matrices were estimated for all specimens altogether (Figure 3) and by location (Figures S4 and S5). All coancestry matrices showed a clear separation between two major groups corresponding to *C. ocellaris* var. *kelberi* and *C. piquiti*. Within groups, *C. piquiti* specimens consistently shared a higher ancestry with one another than *C. ocellaris* var. *kelberi*, indicating greater genetic

homogeneity. Most specimens of the same species from SM and TM did not share the same fine-scale clusters, likely due to intraspecific variation, as previously detected in analyses. The only exception was sample SM31, a putative backcrossed *C. piquiti* hybrid identified by ADMIXTURE analysis. This sample clustered with samples TM6, TM25, and TM31, all of which exhibited evidence of being backcrossed *C. piquiti* hybrids in previous analyses.

Hybrid simulations

Simulations of backcrossed hybrids demonstrated that the ability to distinguish individuals from the parental populations based on ADMIXTURE ancestry proportions decreased with each successive generation of backcrossing. By the fifth generation, all simulated hybrids were indistinguishable from the parental populations, with a probability of > 99% in ADMIXTURE analysis (Fig. S6).

Admixed ancestry and genetic diversity

The relationship between hybrid index and observed heterozygosity showed a clear pattern, with heterozygosity being highest in individuals with ADMIXTURE's posterior probability closer to 0.5 (Figure 4). As hybrid index values deviated from 0.5 toward either parental species, heterozygosity gradually decreased. *C. piquiti* parental specimens (hybrid index > 0.99) or *C. piquiti* backcrosses exhibited lower heterozygosity than *C. ocellaris* var. *kelberi* or *C. ocellaris* var. *kelberi* backcrosses.

D₃ test for introgression

Since introgression signals are evident in the non-native region, we restricted our application of the D₃ test to native populations. Most samples returned significant D₃ values, suggesting widespread introgression (Figure 4). Two samples (SM31 and SM34) displayed significant D₃ values across all possible comparisons, while three samples (SM24, SM32 and SM33) displayed no significant D₃ values when compared to any of the other samples.

Outlier loci and patterns of introgression

Genomic cline analyses based on 16,380 SNPs yielded hybrid indices ranging from 0.002 to 0.988 for hybrid individuals from TM. No β outliers were detected, indicating no evidence for locus-specific differences in the rate of introgression. For α ,

106 outlier loci were identified (0.65% of loci). Among these, 75 loci (0.46%) showed significantly positive α values (*C. ocellaris* var. *kelberi* ancestry), whereas 31 loci (0.19%) showed significantly negative α values (*C. piquiti* ancestry).

Gene Ontology enrichment analysis

Enriched biological processes were predominantly associated with developmental pathways, including pronephros development (GO:0048793), otolith development (GO:0048840), retina development in camera-type eye (GO:0060041), and neural crest cell differentiation (GO:0014033). Signaling-related processes were also represented, notably the ephrin receptor signaling pathway (GO:0048013) and response to cytokine (GO:0034097). Enriched molecular functions included ephrin receptor binding (GO:0046875), potassium channel regulator activity (GO:0015459), and peptidase inhibitor activity (GO:0030414), while the cytosolic large ribosomal subunit (GO:0005882) was the only significantly enriched cellular component.

Discussion

Analysis of genome-wide ddRAD-seq loci from sympatric populations of *C. ocellaris* var. *kelberi* and *C. piquiti* unveiled evident hybrids in the invasive area and subtle signs of introgression in the native populations, highlighting the context-dependent nature of reproductive barriers between these sympatric congeners. This distinction highlights the need to better understand the factors driving introgression and its consequences in these different contexts.

Introgression in native populations

Extensive evidence shows hybridization and introgression between *C. ocellaris* var. *kelberi* and *C. piquiti* in non-native environments (Oliveira et al., 2006; Almeida-Ferreira et al., 2011; Nóbrega et al., 2023). However, in their native range, reports of hybridization were limited to two putative hybrids in the Tucuruí reservoir (Carvalho et al. 2014). This limited number of hybridization records in the native range may be a consequence of previous studies relying exclusively on traditional genetic markers, such as mitochondrial and microsatellite markers (for an overview, see Table 3 in Nóbrega et al. 2023). Despite the effectiveness of these markers in detecting recent hybridization, they may provide limited resolution to capture complex patterns of

introgression, especially when late generation hybrids are prevalent, or when they happened in the past. In our initial ADMIXTURE analyses, a few individuals from Serra da Mesa showed subtle signals of mixed ancestry, however, fastStructure did not detect comparable evidence of admixture in these same individuals. Therefore, to confirm introgression, we used the D_3 method (Hahn & Hibbins 2019), which compares pairwise genetic distances and identifies asymmetries that deviate from a null model of incomplete lineage sorting. D_3 results revealed introgression in the native populations of Serra da Mesa, providing new evidence that hybridization is not confined to non-native environments. Particularly, samples SM31 (*C. piquiti*) and SM34 (*C. ocellaris* var. *kelberi*) consistently showed signatures of introgression regardless of which other samples were included in the D_3 calculation. These samples also showed signals compatible with introgression in other analyses (ADMIXTURE, fineRADstructure). We interpret these outcomes as indicative of relatively recent hybridization events. Based on our simulation results (Figure S4), introgressed individuals become indistinguishable from parental individuals by the fifth backcross generation. Samples SM31 and SM34 ancestry proportions in ADMIXTURE are consistent with simulated fourth generation backcrosses. Considering a generation time of one year (Sastraprawira et al. 2020), this would be consistent with a hybridization event occurring within the last four years.

Despite confirming introgression in a native area, it remains unclear whether *C. ocellaris* var. *kelberi* and *C. piquiti* hybridize under fully natural conditions. Serra da Mesa is a large artificial reservoir created by damming, and reservoirs can substantially alter ecological dynamics by forcing syntopy, modifying spawning habitats, disrupting reproductive cues, and altering hydrological regimes (Juracek 2015; Tang et al. 2022). Such environmental changes may increase the likelihood of hybridization between species that would otherwise remain reproductively isolated in riverine systems (Hasselman et al. 2014; Graham et al. 2020). Recent work suggests that under certain conditions, selection may favor the maintenance of porous species boundaries, particularly when introgressed alleles confer fitness advantages in changing environments (Barraclough 2024). Although we did not directly test for adaptive introgression, this framework provides a plausible explanation for the persistence of introgressed alleles in anthropogenically altered systems. Broader sampling across undisturbed native habitats is necessary to determine whether hybridization occurs under fully natural conditions. If introgression between *C. ocellaris* var. *kelberi* and *C.*

piquiti proves to be recurrent across native populations, it may contribute to regional genomic differentiation, especially within *C. ocellaris* sensu lato. Introgressed alleles could represent an additional source of genetic variation that, under certain ecological conditions, might persist and potentially influence local evolutionary trajectories. (Hedrick 2013; Harrison & Larson 2014).

Role of hybridization in Cichla's invasive success

Hybridization is increasingly recognized as a key driver of invasiveness in both plants and animals, with numerous documented cases where hybrid lineages exhibit enhanced fitness, expanded ecological tolerances, and the ability to invade novel habitats (Schierenbeck & Ellstrand 2009). It can serve as a mechanism that facilitates biological invasions by increasing the adaptive potential of invasive species and mitigating the negative effects of the founder effect (Ellstrand & Schierenbeck 2000; Rius & Darling, 2014). Even in the absence of increased adaptive potential, hybridization with an earlier invader, may help overcome demographic constraints associated with low initial population densities (Gioria et al. 2023). This last scenario is particularly relevant for invasive *Cichla* populations, as frequently, more than one species or varieties are introduced into the same area, as observed in the Três Marias reservoir.

Among freshwater fish, hybridization between invasive species of the genus *Hypophthalmichthys* (carp) is well studied, with research indicating that interspecific hybridization may have played a significant role in facilitating the expansion phase of invasions (Wang et al. 2019). We suggest that a similar dynamic may be at play in *Cichla*, where hybridization could facilitate the establishment of one species in areas where another congeneric is already established. We observed high levels of heterozygosity in invasive TM populations, especially among hybrid individuals. This same pattern of high genetic diversity is observed in other invasive peacock bass populations in which hybridization is ongoing (Oliveira et al. 2006; Almeida-Ferreira et al. 2011; Diamante et al. 2020; Nóbrega et al. 2023). Although high genetic diversity at neutral loci does not necessarily indicate an adaptive advantage (Teixeira & Huber 2012), alleles that are neutral under one set of environmental conditions may become beneficial if selective pressures shift (de Lafontaine et al. 2018; Chung et al. 2023), which is particularly relevant for widespread invasive species. Furthermore, the distinction between neutral and adaptive loci in these species remains unclear due to

limited genomic knowledge, making it difficult to determine which genetic variants contribute to adaptation and which are maintained by drift.

It is also possible that pre-introduction hybridization events created source populations already carrying introgressed genomic combinations that facilitated their establishment (Laikre et al. 2010; Gaskin 2017). Post-introduction hybridization in the invasive range may then further enhanced genetic diversity and ecological breadth, accelerating establishment and spread. While the adaptive significance of these patterns remains to be fully elucidated, the consistent association between hybridization, high genetic diversity and invasive success in *Cichla* supports the view that introgression may be an important mechanism in their range expansion. This is further emphasized by recent work showing *C. ocellaris* var. *kelberi* reproductive and trophic plasticity in invaded habitats (Gaspar et al. 2024). Although that study did not examine hybridization directly, the presence of hybridizing congeners (*C. piquiti* and *C. ocellaris* var. *monoculus*) in several of the sampled reservoirs (e.g., Lajeado, Santa Cruz River, and Juturnaíba) raises the possibility that introgression may be contributing to the observed ecological flexibility.

Patterns of introgression

Directional introgression occurs when one species is more likely to mate with hybrids or when hybrids preferentially backcross with one parental species, leading to unidirectional gene flow. This can result from differences in mating preferences, hybrid viability, or reproductive success. In contrast, locus-specific asymmetry in introgression refers to variation in how different genomic regions introgress, with some loci being freely exchanged while others are restricted due to selection (Martin & Jiggins 2017). This genomic heterogeneity is influenced by factors such as selection against deleterious alleles, adaptive introgression, and the recombination landscape, which determines whether certain regions act as barrier loci that resist gene flow or porous regions where introgression is more likely (Harrison & Larson 2014). Our analyses indicate that introgression occurs in both directions, although more frequently in the direction of *C. ocellaris* var. *kelberi*, consistent with demographic asymmetry in gene flow. At the genomic level, the bayesian genomic cline analysis conducted in Três Marias, 106 α outliers were detected, with 75 loci showing significantly positive α values and 31 showing significantly negative α values. This pattern indicates locus-specific deviations from neutral introgression rather than strictly directional gene flow

between species. Some genomic regions show a bias toward *C. ocellaris* var. *kelberi* ancestry, whereas others are biased toward *C. piquiti* ancestry, indicating heterogeneous patterns of locus-specific introgression.

Therefore, the higher genetic diversity observed in *C. ocellaris* var. *kelberi* is more consistent with asymmetric backcrossing than with locus-specific ancestry alone. Directional introgression into *C. ocellaris* var. *kelberi* provides repeated opportunities for the incorporation of novel alleles, increasing overall genetic diversity. Nonetheless, demographic history and differences in effective population size remain plausible alternative explanations for this pattern. Gene Ontology enrichment analyses of α outlier loci identified several overrepresented functional categories, although most terms were broad. These results provide preliminary insight into potential functional relevance but do not allow definitive conclusions regarding adaptive introgression. Further investigation is required to identify genomic regions under selection and to clarify their evolutionary significance.

Breakdown of reproductive isolation mechanisms

Hybridization resulting from species translocations is often linked to the disruption of geographic isolation, but it can also occur between naturally coexisting species (e.g., Cummins et al. 2023). This may be driven by ecological changes, such as forced syntopy due to niche limitations in new areas, imbalances in population sizes between species (Hubbs' "desperation hypothesis") (Hubbs 1955), environmental changes causing temporal overlap in reproductive cycles, or disruption of reproductive cues. Although both Serra da Mesa and Três Marias experienced similar human-induced changes (dam construction), they are located in different freshwater ecoregions; therefore, they differ in dynamics, environmental conditions, and species composition. The fact that hybrids are pervasive in many introduced populations, despite being rare in native populations, strongly suggests prezygotic reproductive isolation (ecological or behavioral) as the primary mechanism of isolation between *C. ocellaris* var. *kelberi* and *C. piquiti*. The lack of early generation hybrids in Serra da Mesa and the overall lack of hybridization records in other reservoirs in the native areas suggest that natural reproductive isolation mechanisms are still mostly intact despite the anthropogenic alterations related to river damming.

Few studies have investigated the ecological traits that could serve as reproductive barriers between sympatric *C. ocellaris* var. *kelberi* and *C. piquiti* in their

native range. In the Lajeado reservoir (native), yellow and blue peacock bass share many functional traits and behavioral aspects, including habitat occupation and diet (Andrade & Pelicice 2022). However, the study revealed that the reproductive periods differ markedly across species: *C. piquiti* reproduces mostly between January and July, and *C. ocellaris* var. *kelberi* reproduces primarily between September and December. In the Três Marias reservoir (invaded), Normando et al. (2009) found that *C. ocellaris* var. *kelberi* showed reproductive activity throughout the whole year, with peaks between September and October. In the Itumbiara reservoir (invaded), *C. piquiti* shows reproductive activity during almost the entire year, with a reproductive peak prior to the beginning of rains (August/September) (Vieira et al. 2009). The expanded reproductive window in non-native areas is hypothesized to be associated with their successful establishment (Garcia et al. 2018). It could also lead to increased hybridization rates, while in their native range, the distinct breeding seasons likely act as a reproductive barrier.

Another potential factor contributing to higher introgression rates in introduced *Cichla* populations is the imbalance in species abundance, known as Hubbs' Principle (Hubbs 1955). In reservoirs where multiple peacock bass species are introduced, one species is typically introduced first. With the introduction of a second species, an imbalance naturally occurs, with the newly introduced species being significantly outnumbered by the already established one. Due to female choice, it is also expected that females of the rarer species will eventually mate with heterospecific males from the more common species when conspecific males become scarce (Wirtz 1999). This scenario can also bias introgression toward the more abundant species, as F1 hybrids are more likely to backcross with them. In areas such as the Três Marias reservoir, the Ribeirão das Lajes reservoir (Nóbrega et al. 2023), and the Paraná River Basin (Almeida-Ferreira et al. 2011; Carvalho et al. 2014), where introgression is frequent, *C. ocellaris* var. *kelberi* was already established before the introduction of *C. piquiti*. However, while numerical imbalance may increase the probability of heterospecific encounters, it does not fully explain the relaxation of behavioral or ecological barriers required for successful interspecific mating. Therefore, other factors must play a role in the increased hybridization rates in introduced populations. The assessment of environmental characteristics across areas with different introgression rates could indicate which factors are more important in the breakdown of reproductive barriers between *Cichla* species.

Future directions

This study provides insights into the patterns and processes of hybridization between *C. ocellaris* var. *kelberi* and *C. piquiti*, but it also raises important questions that warrant further investigation. Broader geographic sampling across the native range, particularly from undisturbed riverine systems, is needed to assess baseline levels of natural introgression and clarify whether hybridization in their native range is truly rare or underdetected. Experimental and behavioral studies could help elucidate the mechanisms of reproductive isolation, including mate choice, spawning behavior, and reproductive timing under different environmental conditions. Furthermore, the functional consequences of introgression to fitness remain poorly understood. Future work could provide a better understanding of how introgression shapes phenotypic traits such as morphology, behavior, or ecological tolerance, and its role in the ecological success of invasive populations.

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Figures

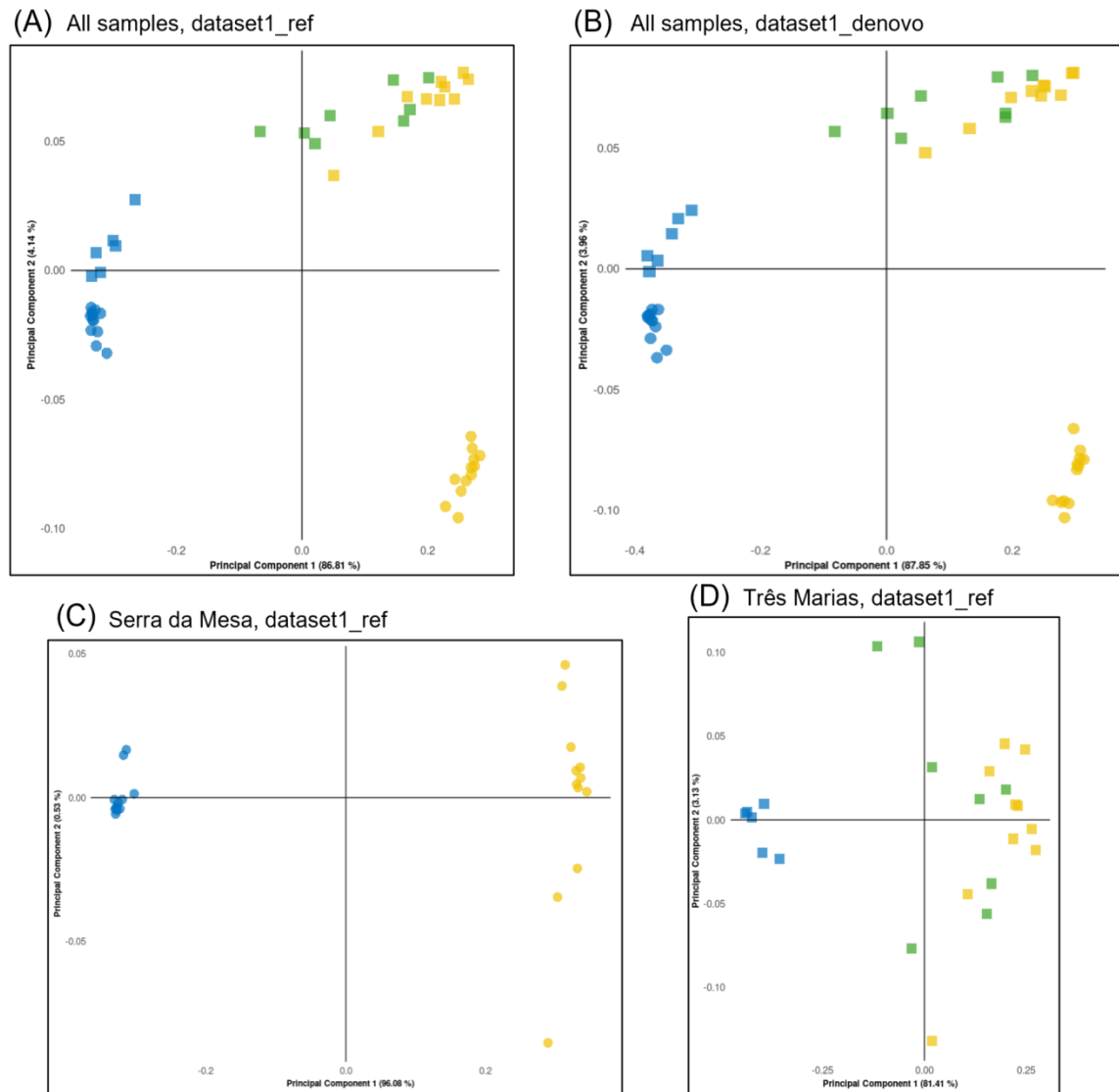


Figure 1. Principal Component Analysis (PCA) of *Cichla* samples from Serra da Mesa (SM) and Três Marias (TM) reservoirs. PCA of all SM and TM samples using (A) dataset1_ref and (B) dataset1_denovo. (C) PCA of only SM samples using dataset1_ref. (D) PCA of only TM samples using dataset1_ref. SM specimens are identified by circles and TM specimens by squares. Blue represents specimens morphologically identified as *C. piquiti*, yellow as *C. ocellaris* var. *kelberi* and green as putative hybrids.

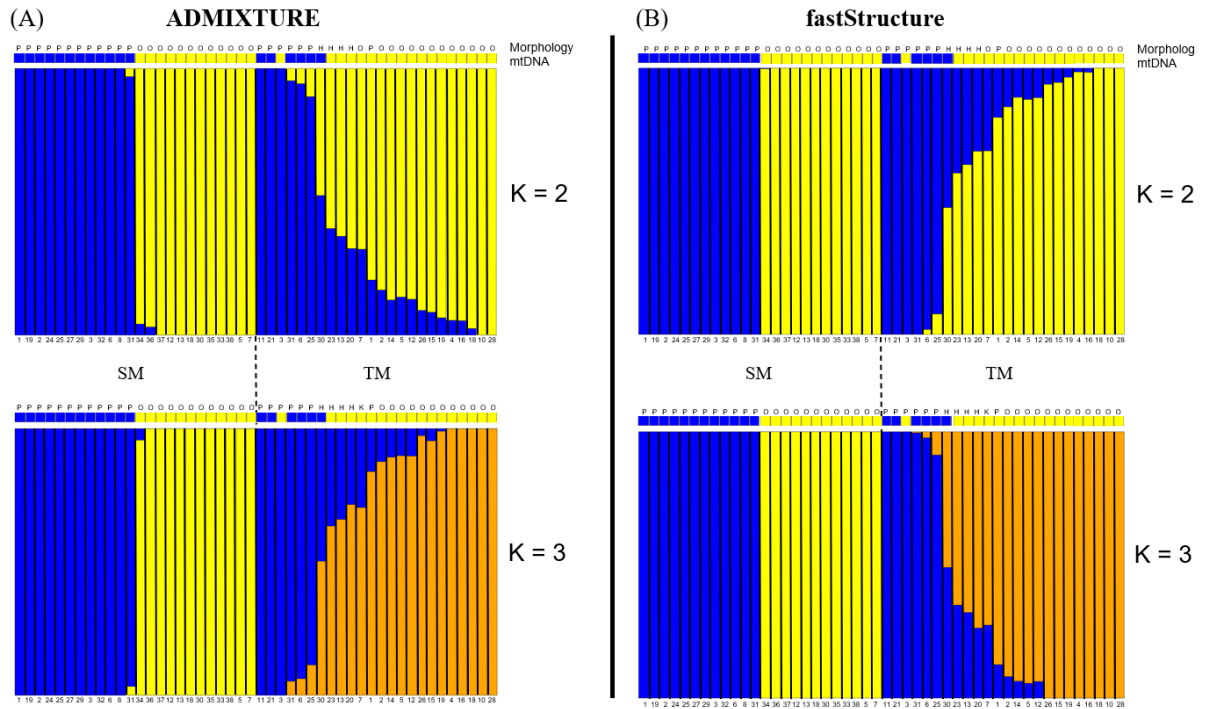


Figure 2. Morphological identification, mtDNA Control Region haplotypes and barplots, based on dataset2_ref, from ADMIXTURE (A) and fastStructure (B) results at K = 2 and 3. In the morphology session O, P and H indicates *C. ocellaris* var. *kelberi*, *C. piquiti* and hybrids, respectively. In the mtDNA session, yellow rectangles indicate *C. ocellaris* var. *kelberi* haplotypes and blue indicates *C. piquiti* haplotypes. Each individual is represented by a vertical bar.

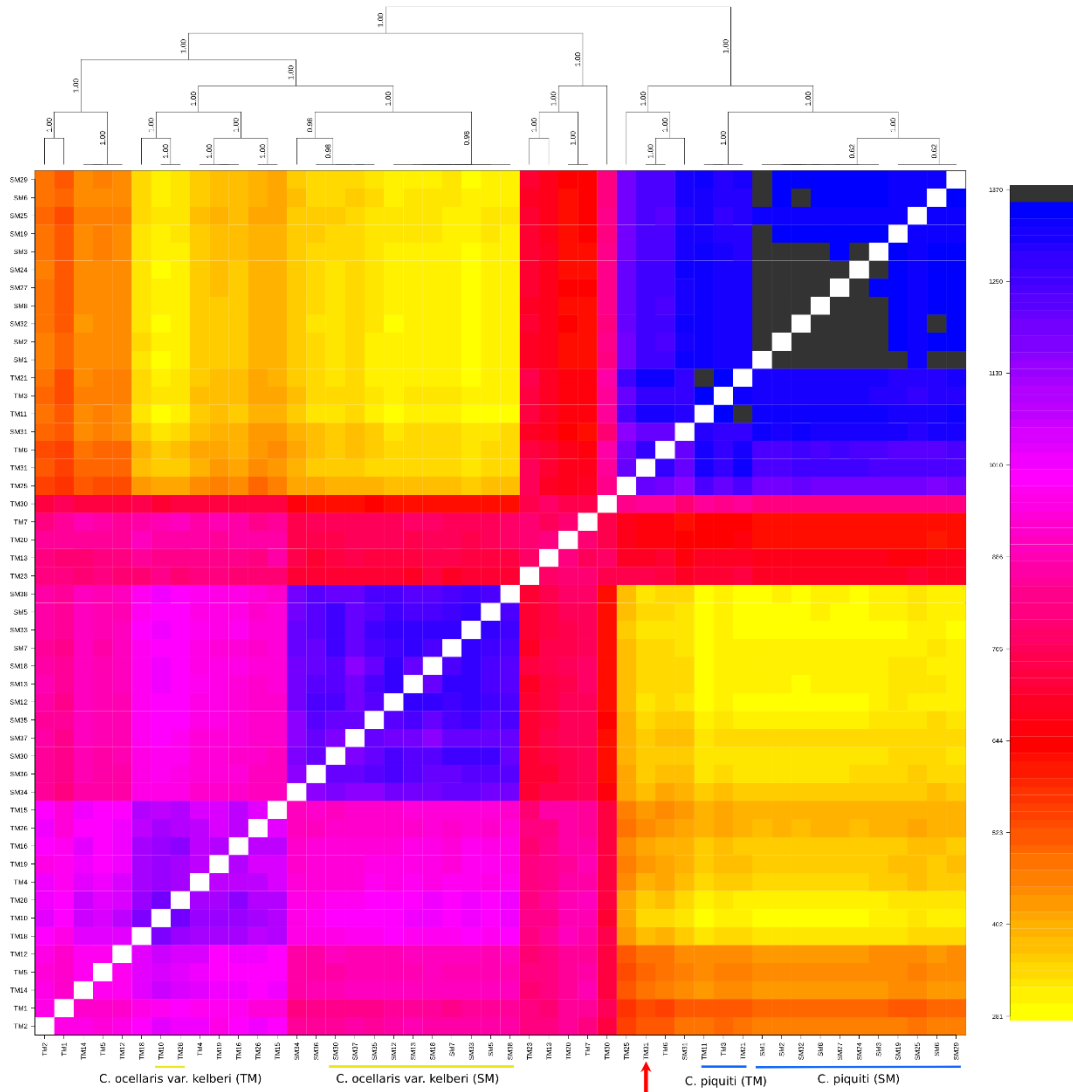


Figure 3. fineRADstructure coancestry matrix of all sampled individuals. Black and yellow indicate maximum and minimum levels of coancestry between two individuals, respectively. Samples from each area with ≥ 0.99 ancestry in the ADMIXTURE analysis are marked by a colored bar beneath them (yellow for *C. ocellaris* var. *kelberi*, blue for *C. piquiti*). A red arrow denotes the only Serra da Mesa sample that clustered with individuals from Três Marias.

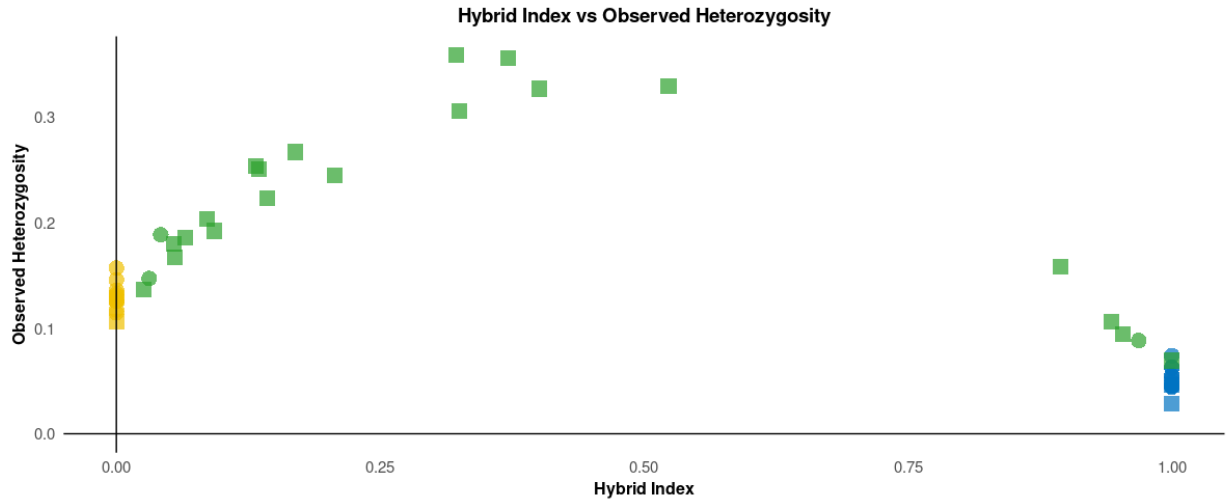


Figure 4. Plot showing the relationship between observed heterozygosity and ADMIXTURE's hybrid index for each individual. SM specimens are identified by circles and TM specimens by squares. Blue represents specimens identified as *C. piquiti*, yellow as *C. ocellaris* var. *kelberi* and green as hybrids, based on a threshold of 0.99 in ADMIXTURE's ancestry fraction (hybrid index) to identify pure specimens.

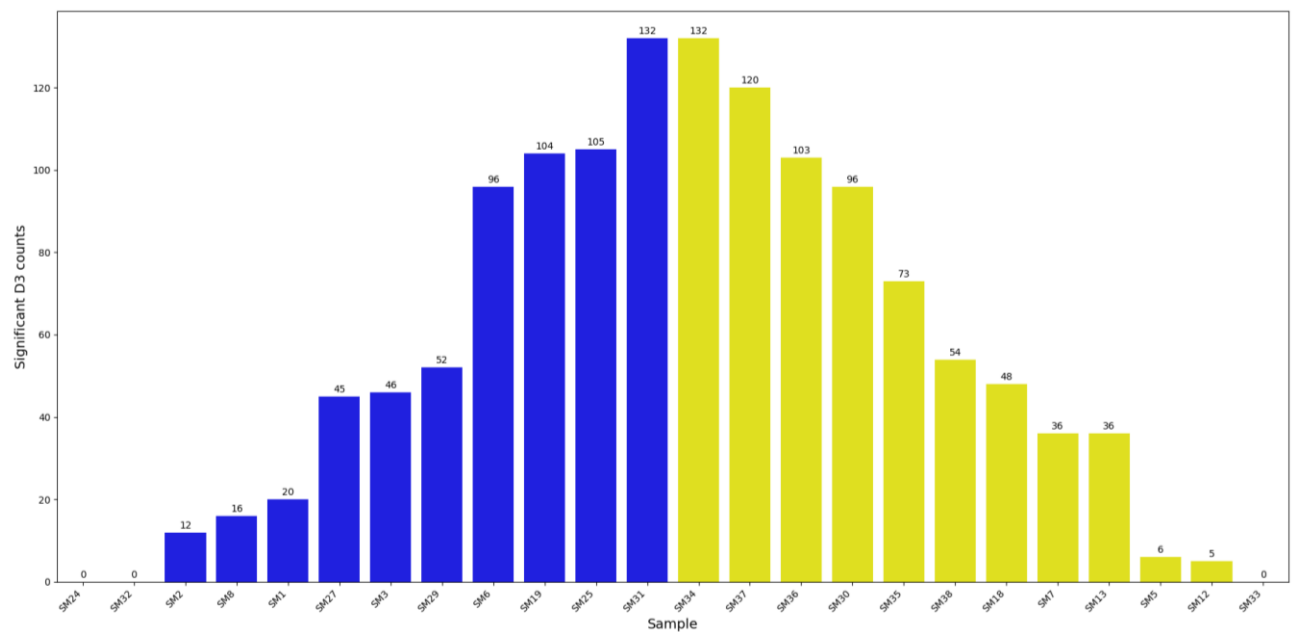


Figure 5. Bar plot of the number of significant D3 results per sample, considering each specimen as sample A. *C. ocellaris* var. *kelberi* in yellow, *C. piquiti* in blue

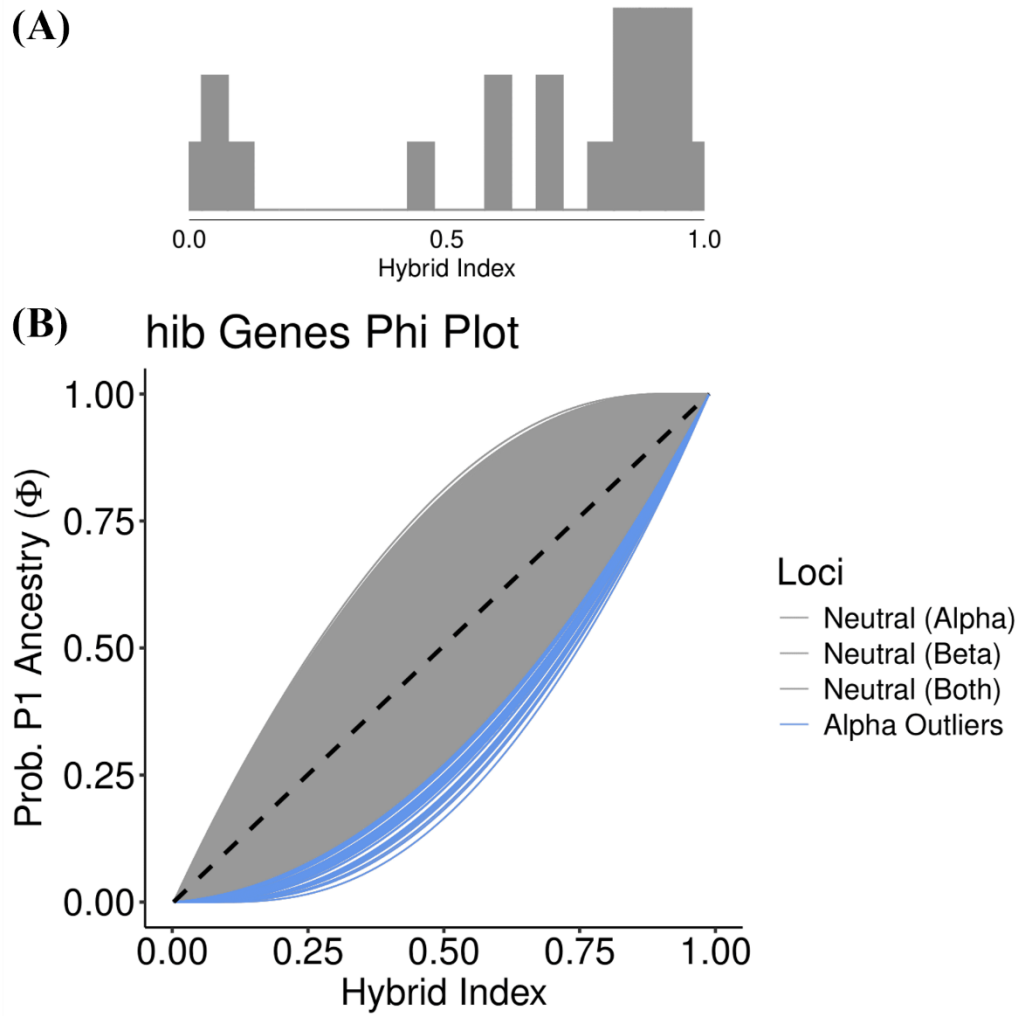
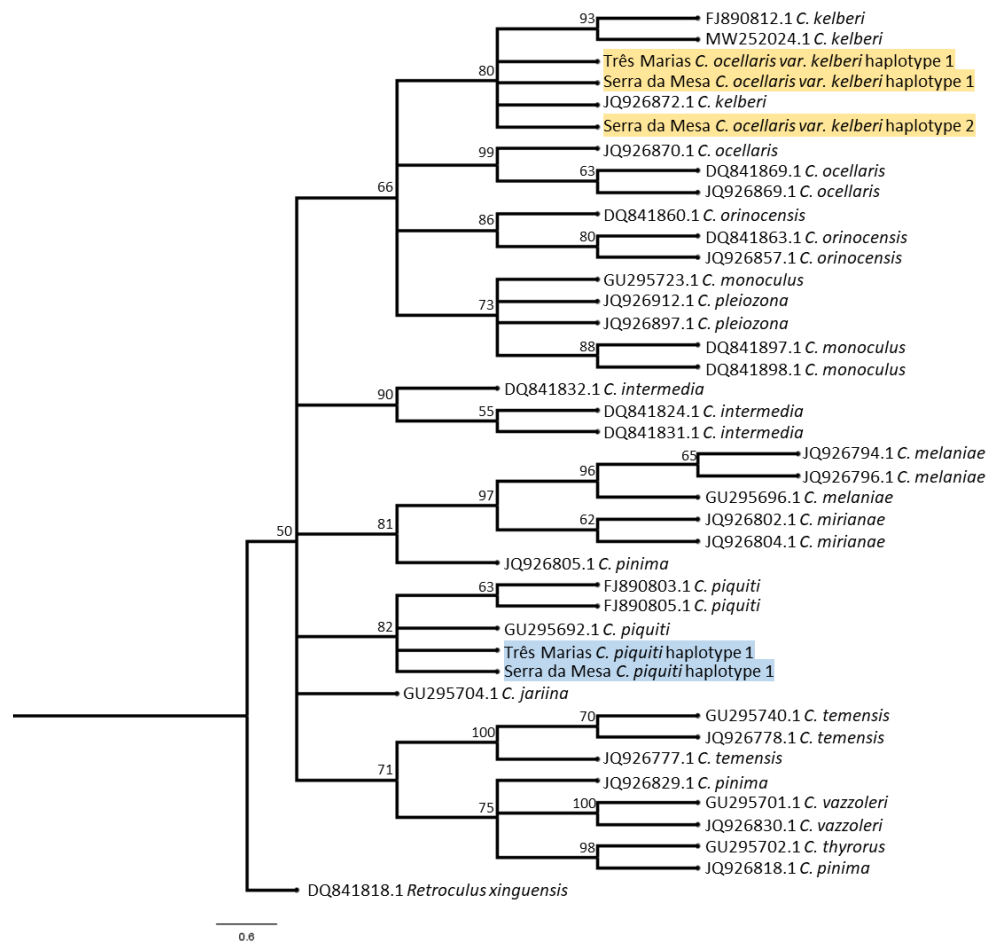
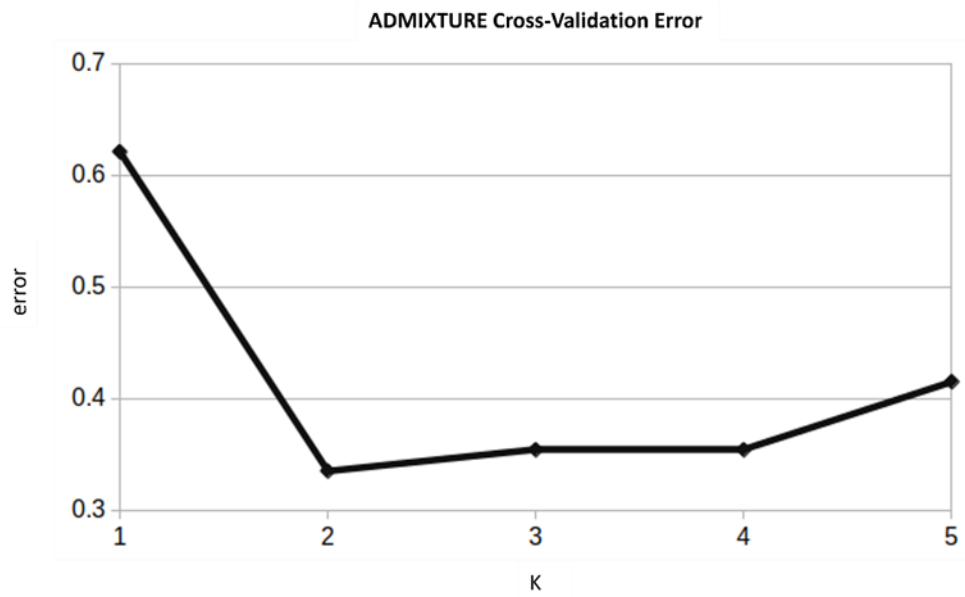


Figure 6. Results of a Bayesian genomic cline analysis in bgc. (A) Histogram of distribution of hybrid indices (B) Probability of *C. piquiti* ancestry (Φ) as a function of the hybrid index. The shaded area represents the expected genomic cline for neutral loci, with alpha outlier loci shown in blue.

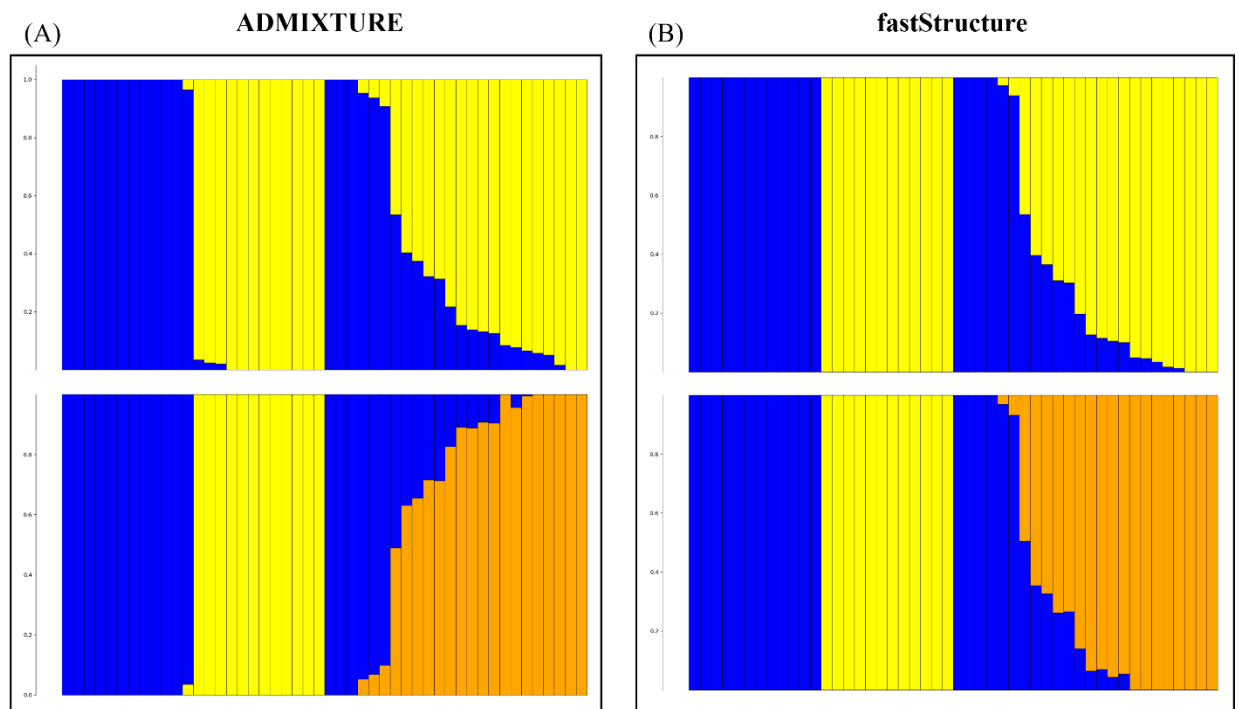
Supplementary Figures



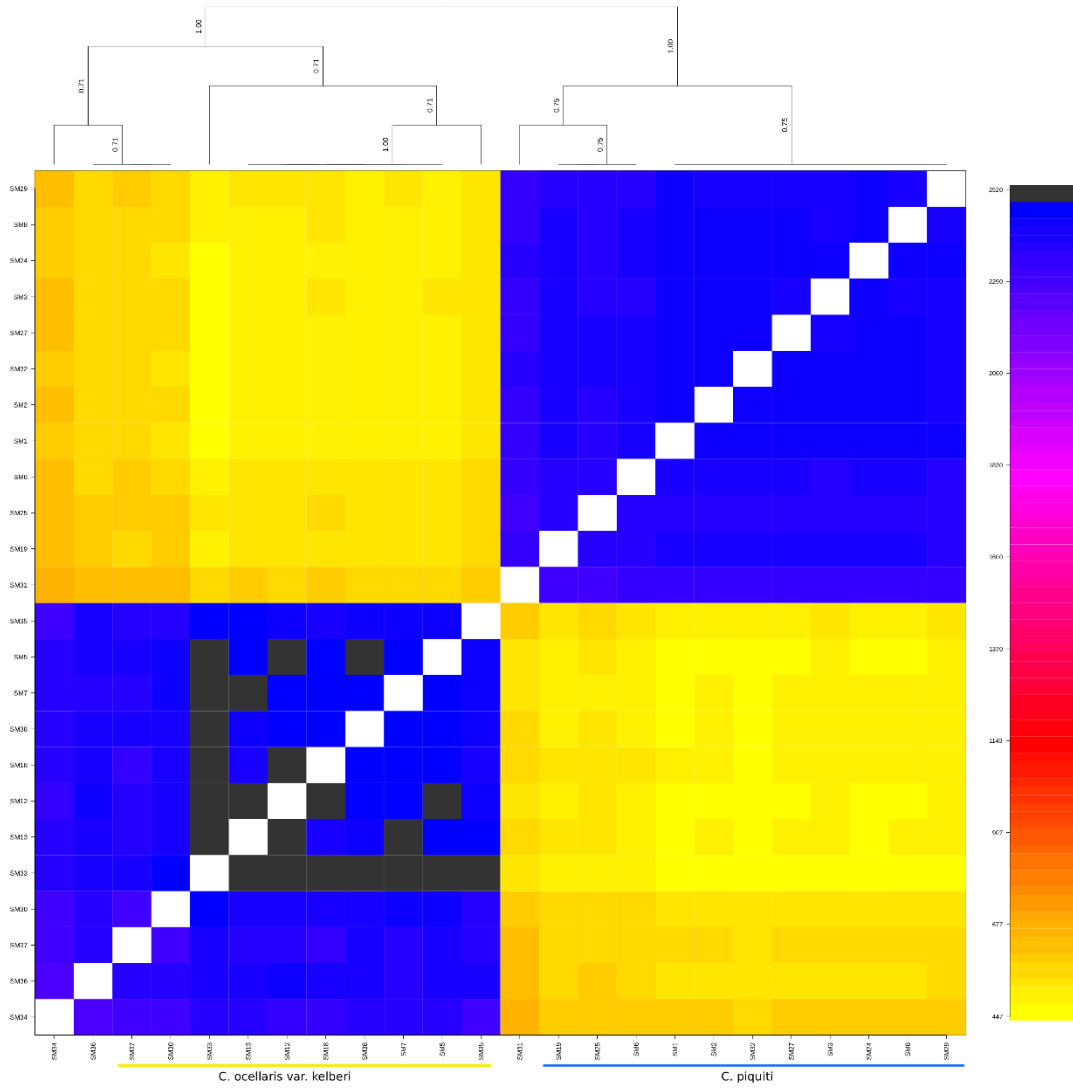
Supplementary Figure 1. Maximum Likelihood tree based on Control Region sequences of *Cichla* species captured at Serra da Mesa and Três Marias and others taken from GenBank. Numbers above nodes represent bootstrap values



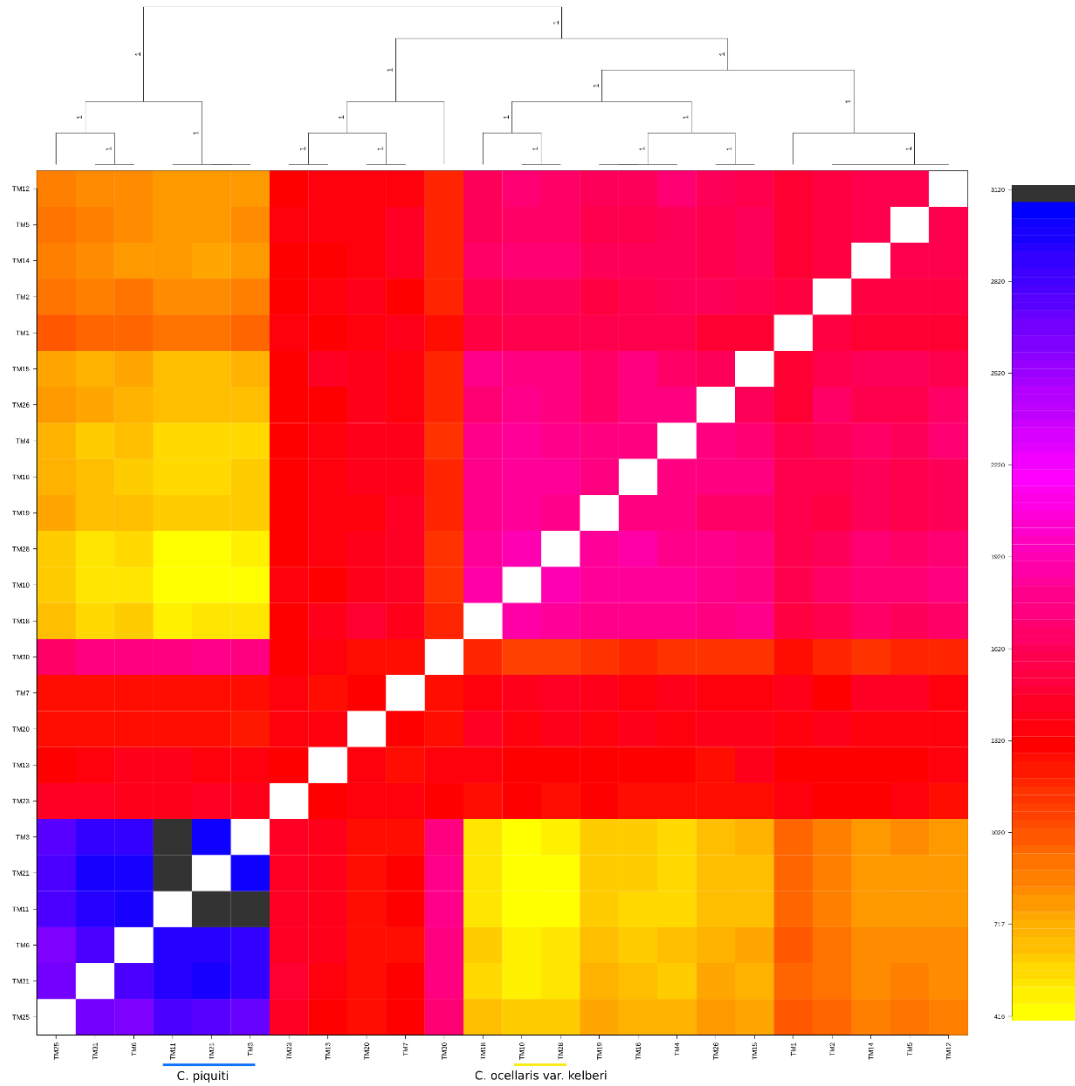
Supplementary Figure 2. Cross-validation error obtained from ADMIXTURE runs for $K = 1-5$



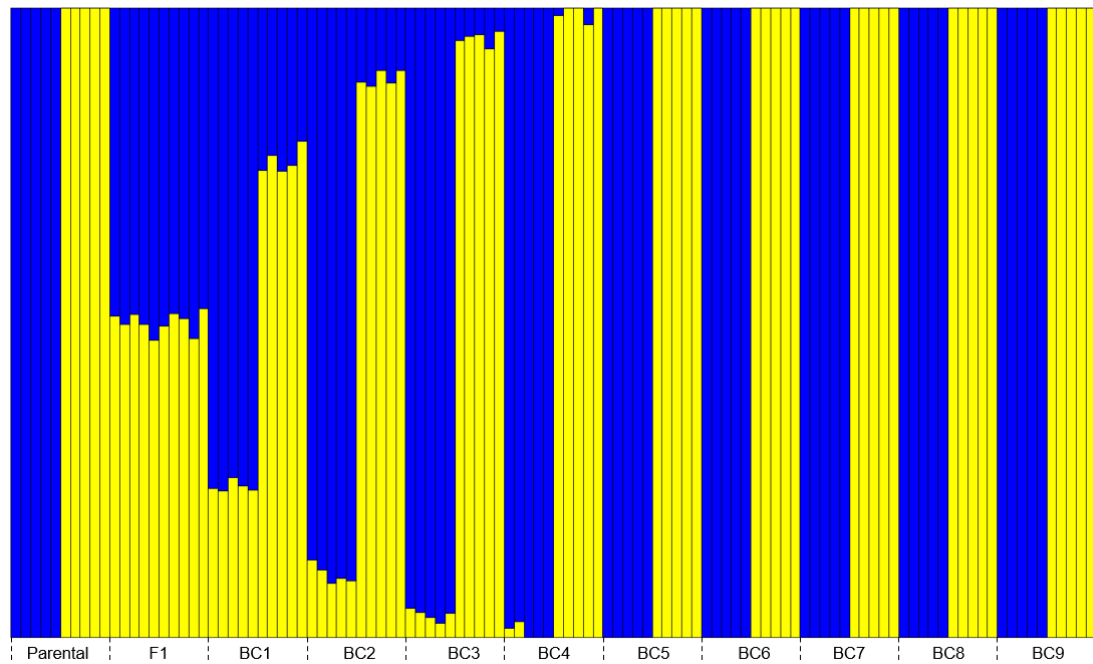
Supplementary Figure 3. Barplots, based on dataset2_denovo, from ADMIXTURE (A) and fastStructure (B) results at $K = 2$ and 3 . Each individual is represented by a vertical bar. Blue = *C. piquiti* ancestry; yellow and orange = *C. ocellaris* var. *kelberi* ancestry



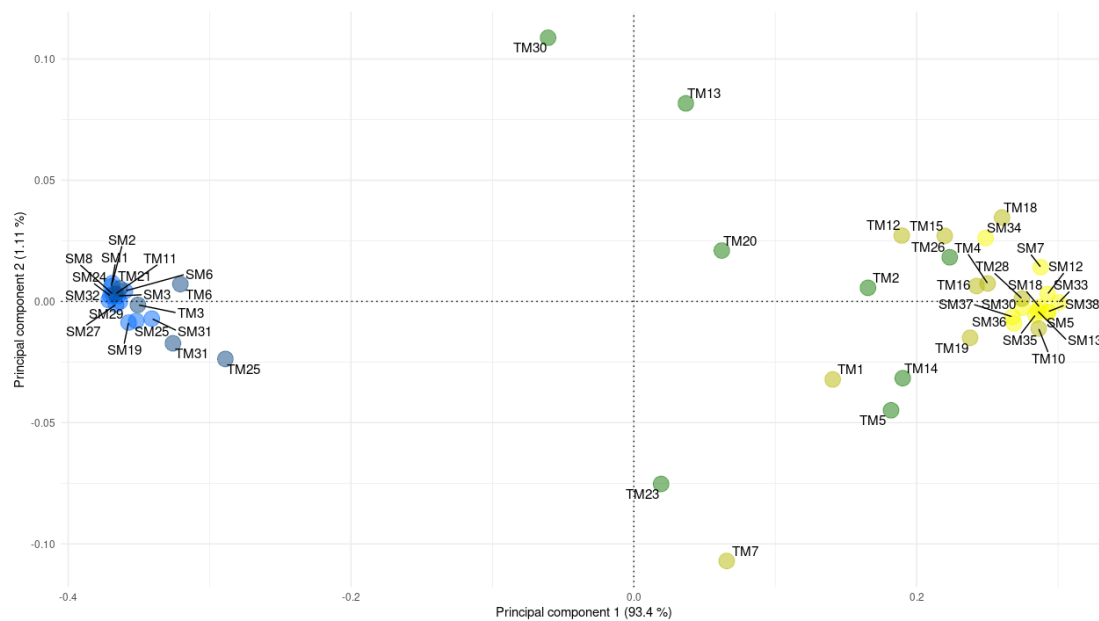
Supplementary Figure 4. fineRADstructure coancestry matrix of samples from Serra da Mesa. Black and yellow indicate maximum and minimum levels of coancestry between two individuals, respectively. Samples with ≥ 0.99 ancestry in the ADMIXTURE analysis are marked by a colored bar beneath them (yellow for *C. ocellaris* var. *kelberi*, blue for *C. piquiti*).



Supplementary Figure 5. fineRADstructure coancestry matrix of samples from Três Marias. Black and yellow indicate maximum and minimum levels of coancestry between two individuals, respectively. Samples with ≥ 0.99 ancestry in the ADMIXTURE analysis are marked by a colored bar beneath them (yellow for *C. ocellaris* var. *kelberi*, blue for *C. piquiti*).



Supplementary Figure 6. Barplot from ADMIXTURE analysis based on the simulated backcrossing between parental *C. ocellaris* var. *kelberi* and *C. piquiti*.



Supplementary Figure 7. PCA of filtered SNP dataset for bgc analysis showing minimal differentiation between species from each area.

Supplementary Tables

Supplementary Table 1. Summary of loci and SNP recovery across reference-based and de novo pipelines. *r* – minimum locus presence across samples; min-maf – minor allele frequency threshold; write-single-snp – limits to one SNP per locus

Dataset	Loci discovery method	Parameters	Loci	SNPs
dataset1_ref	Referenced-based	-r 0.8 --min-maf 0.02	96774	48589
dataset2_ref	Referenced-based	-r 0.8 --min-maf 0.02 --write-single-snp	96774	34923
dataset1_denovo	De novo	-r 0.8 -t 8 --min-maf 0.02	261333	272555
dataset2_denovo	De novo	-r 0.8 --min-maf 0.02 --write-single-snp	261333	151989

Capítulo 3 — Morphological intermediacy in hybrid *Cichla* (*C. ocellaris* var. *kelberi* × *C. piquiti*) is strongly correlated with genomic admixture.

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Keywords: geometric morphometrics; hybridization; invasive species

Abstract

Hybridization is a key evolutionary process, which can have significant phenotypic consequences. In South American reservoirs, the introduced peacock bass *Cichla ocellaris* var. *kelberi* and *Cichla piquiti* are known to hybridize, but the morphological outcomes of this admixture remain largely unexplored. This study integrates genomic and morphometric data to characterize the phenotypic results of this hybridization. We used geometric morphometrics on eight landmarks from 44 individuals, whose genetic status (parental or hybrid) was a priori determined by ddRAD-seq SNP data. We aimed to quantify shape differences between parental species and hybrids, test the association between morphological variation and genomic ancestry (Hybrid Index, HI), and compare morphological disparity among groups. Procrustes ANOVA revealed significant morphological divergence between the parental species, with *C. ocellaris* var. *kelberi* possessing a deeper body and broader head, while *C. piquiti* being more streamlined with a narrower head. Hybrid individuals occupied an intermediate morphological position. Canonical Variate Analysis (CVA) confirmed this clear separation, and we found a strong, significant correlation ($r = -0.893$, $P < 0.001$)

between the primary axis of morphological shape (CV1) and the genomic Hybrid Index. This indicates that an individual's body shape is a reliable predictor of its genomic ancestry. Furthermore, an analysis of morphological disparity showed no significant difference in variance between hybrids and parental species, suggesting that hybridization primarily results in intermediate, additive phenotypes rather than novel or transgressive forms. Our findings demonstrate that hybridization between these *Cichla* species produces a predictable morphological gradient tightly linked to genomic introgression. We suggest that while the distinct parental ecomorphologies are likely adaptive in their native, structured riverine habitats, the intermediate hybrid phenotypes are not selected against in non-native environments, facilitating the success of hybrid swarms.

Introduction

Hybridization and introgression play significant roles in the evolution and diversification of species, influencing both genetic and phenotypic variation (Mallet, 2005; Abbott et al., 2013). In fishes, hybridization has been widely documented, often resulting in intermediate or novel phenotypes that can affect fitness, ecological interactions, and species boundaries (Seehausen, 2004; Nolte & Sheets, 2005). Understanding the morphological and genetic outcomes of these events is therefore essential for elucidating the dynamics of species boundaries, adaptation, and biodiversity maintenance. In parallel, morphology remains one of the most accessible and ecologically relevant dimensions of organismal diversity. Morphological traits are often tightly linked to ecological performance, resource use, and reproductive success, making them key indicators of both evolutionary divergence and hybridization (Clabaut et al. 2007; Leal et al. 2011; Parsons et al. 2003). When hybridization occurs, morphological variation may reflect underlying genetic admixture, producing individuals with intermediate, mosaic, or even transgressive phenotypes (McBride & Singer 2010; Parson et al. 2011). Morphometric analyses provide a powerful approach to understanding how hybridization influences phenotypic variation. Traditional morphometrics and geometric morphometrics allow for the quantitative assessment of morphological differences between species and their hybrids, offering insights into patterns of trait inheritance and selection (Zelditch et al., 2012; Parson et al. 2011). In particular, geometric morphometrics, which uses landmark-based methods to analyze

shape variation, has been widely applied to study hybrid morphology in fish populations (Parsons et al., 2003; Kelly et al. 2025).

The Neotropical peacock basses of the genus *Cichla* (Cichlidae) offer a valuable model to explore these questions. Native to South America, several *Cichla* species have been introduced into reservoirs and river basins mainly for sport fishing, where they have become invasive and caused major changes in the local ecosystems (Zaret and Paine 1973; Pelicice & Angelo 2009; Ota et al. 2019; Franco et al. 2021a). Among them, *Cichla piquiti* and *Cichla ocellaris* var. *kelberi* are of particular interest because they co-occur naturally in the Tocantins–Araguaia basin but were also jointly introduced into multiple artificial reservoirs outside their native range (Franco et al. 2021b; Nóbrega et al. 2023). In these non-native environments, hybridization between both species has been reported, producing fertile hybrids and admixed populations (Oliveira et al., 2006; Almeida-Ferreira et al., 2011; Nóbrega et al., 2023). However, despite growing molecular evidence for hybridization, its phenotypic consequences remain largely unexplored.

Morphological analyses of *Cichla* hybrids are particularly relevant for several reasons. First, *Cichla* species are morphologically similar and traditionally diagnosed based on coloration patterns and morphometrics (Kullander & Ferreira 2006), traits that may be affected by hybridization. Second, morphology can influence ecological performance and habitat use, meaning that hybrid forms could differ in feeding strategy, swimming efficiency, or reproductive behavior. Finally, understanding whether hybrid individuals display intermediate or biased phenotypes relative to parental species can provide insights into the direction and stability of introgression in natural populations.

In this study, we investigate the morphological variation in *C. ocellaris* var. *kelberi* and *C. piquiti* and their hybrids using geometric morphometric. All specimens were previously identified based on ddRAD-seq by Nobrega et al. (in prep.), providing a robust genetic framework for assessing phenotypic differences. We integrate morphometric and genetic data to evaluate whether hybrid individuals exhibit intermediate, parental-biased, or transgressive morphological traits, and whether morphological patterns are consistent with genomic ancestry proportions. Specifically, we aimed to (i) quantify overall shape variation among parental species and hybrids; (ii) evaluate whether hybrid morphology reflects their genomic composition; and (iii) assess the degree of morphological overlap between hybrids and parental species. By linking morphological variation with genomic background, this study provides insights

into the phenotypic outcomes of hybridization in *Cichla* and contributes to a broader understanding of how introgression shapes organismal form in invasive fish populations.

Materials and Methods

Sampling

A total of 24 individuals were sampled from the Serra da Mesa reservoir (SM, 13° 58' 58.1376" S, 48° 21' 48.942" W), and 20 individuals from the Três Marias reservoir (TM, 18° 35' 57.3288" S, 45° 14' 10.6728" W), totaling 44 samples. Serra da Mesa showed minimal evidence of hybridization, with most individuals identified as genetically pure *C. piquiti* or *C. ocellaris* var. *kelberi*. In contrast, Três Marias has a hybrid population, where pure parental specimens are rare. Fish were identified using the morphological characterization established by Kullander & Ferreira (2006). Photographs of *Cichla* specimens in lateral view were taken in the field using a digital camera. Each specimen was positioned with its lateral side facing up, alongside a ruler for scale, and the camera lens was aligned parallel to the sagittal plane to minimize distortion. The genetic ancestry and hybrid status of all individuals were determined from a ddRAD-seq dataset comprising 34,923 SNPs (Nóbrega et al., in prep.). Ancestry coefficients were estimated in ADMIXTURE, and individuals with assignment values ≥ 0.99 to a single cluster were classified as parental. Individuals with intermediate values were classified as hybrids. Based on these criteria, samples from SM consisted of 10 *C. ocellaris* var. *kelberi*, 11 *C. piquiti*, and three hybrids. Samples from TM consisted of two *C. ocellaris* var. *kelberi*, three *C. piquiti*, and 15 hybrids. These genetically defined categories were used as a priori groups in the morphometric analyses

Data acquisition

Raw images were compiled into a TPS file using tpsUtil32 (Rohlf, 2015). Using tpsDig 2.32 (Rohlf, 2015), two types of morphometric data were collected from each photograph. First, eight homologous landmarks were digitized using x- and y-coordinates. Landmarks were selected based on prior studies demonstrating their effectiveness in capturing fish body shape (Loy et al., 2000; Cunha et al., 2009; Tancioni et al., 2013). These digitized coordinates were subjected to Generalized

Procrustes Analysis (GPA) to eliminate variation due to size, position, and orientation, preserving only shape-related information. Second, a set of six linear measurements was also recorded: standard length, eye diameter, head depth, peduncle depth, head length, and body depth.

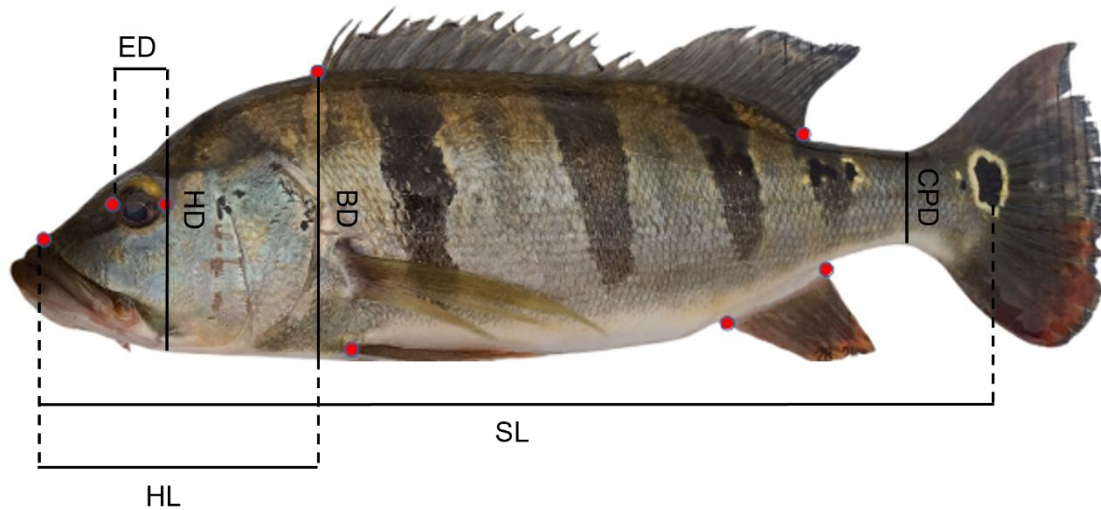


Figure 1. Homologous landmarks positions (red dots) and morphometric characters on a *Cichla* specimen: standard length (SL), head length (HL), head depth (HD), body depth (BD), eye diameter (ED), caudal peduncle depth (CPD).

Assessment of measurement error

Assessing measurement error (ME) is essential in morphometric studies, particularly when landmark data are obtained from photographs taken under field conditions, where variable angles, lighting, and specimen positioning can introduce error. To quantify ME, each specimen was digitized twice, providing two independent landmarking replicates per individual. ME was evaluated using the ME-ANOVA implemented in the geomorph package (function `gm.measurement.error()`), which partitions total ME into random and systematic components (Collyer and Adams 2024). In this framework, random ME reflects inconsistent landmark placement between replicates, whereas systematic ME (bias) represents consistent directional deviations. The analysis compares individual variation and systematic ME to random ME using signal-to-noise ratios (SNRs), with significance assessed via 999 permutations of model residuals.

Data analyses

To quantify morphological variation among individuals and groups, five ecomorphological indices were calculated based on the standard linear measurements: relative eye width (ED/SL), relative head length (HL/SL), relative head depth (HD/SL), relative caudal peduncle depth (CPD/BD), body depth index (BH/SL). Differences among groups (*C. ocellaris* var. *kelberi*, *C. piquiti*, and their hybrids) were tested using one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc tests when significant effects were detected.

All geometric morphometric analyses were conducted using the geomorph package in R (Adams & Otárola-Castillo, 2013). The influence of allometry (the effect of size on shape) was assessed by regressing Procrustes shape coordinates against centroid size using Procrustes linear models (procD.lm function). The significance of size effects on shape was tested with 999 permutations. As no significant allometric effect was detected ($p = 0.141$), shape analyses were conducted without size correction.

To test for shape differences among the three groups predefined by the genetic analysis (*C. ocellaris* var. *kelberi*, *C. piquiti*, and hybrids), a Procrustes ANOVA was applied using 999 permutations. Post hoc pairwise comparisons were performed to identify specific group differences. The mean shapes were then plotted together in a wireframe overlay to visually represent the principal shape differences between groups.

Principal Component Analysis (PCA) was performed on Procrustes-aligned coordinates to summarize overall shape variation without prior grouping. Canonical Variate Analysis (CVA) was then used to maximize separation among groups based on shape variables.

To test and compare the association between morphology and genetic ancestry across both morphometric methods, two parallel Pearson correlation analyses were performed. First, for the geometric morphometric data, we correlated individual scores from the first canonical variate (CV1) of the CVA with the genomic Hybrid Index (HI) for each individual. Second, for the traditional linear measurements, an analogous analysis was conducted. A Linear Discriminant Analysis (LDA) was first performed on the size-corrected linear ratios to generate a single axis of morphological differentiation (LD1). These LD1 scores were then correlated with the HI. This dual approach allowed

for a direct comparison of the strength of the morphogenetic signal captured by each method.

Finally, to compare the amount of morphological variation within each group, a Morphological Disparity analysis was conducted using the geomorph function `morphol.disparity()`. This analysis calculates the Procrustes variance for each group (hybrids, *C. ocellaris* var. *kelberi*, and *C. piquiti*) and tests for significant differences in these variances through pairwise comparisons with 999 permutations.

Results

Linear morphometrics

Mean values for the linear morphometric traits differed moderately among the three groups (Table 1). Hybrids tended to be larger overall, showing the greatest standard length (36.23 cm) and the highest mean values for head depth, caudal peduncle depth, head length, and body depth. *C. ocellaris* var. *kelberi* and *C. piquiti* exhibited similar standard lengths, but differed in some traits: *C. ocellaris* var. *kelberi* showed a larger eye diameter (1.73 cm), whereas *C. piquiti* displayed slightly greater caudal peduncle depth (3.65 cm).

Table 1. Mean linear morphometric measurements for *C. ocellaris* var. *kelberi*, *C. piquiti*, and hybrids. Values represent group means for standard length (SL), eye diameter (ED), head depth (HD), caudal peduncle depth (CPD), head length (HL), and body depth (BD).

Group	SL	ED	HD	CPD	HL	BD
<i>C. ocellaris</i>						
var. <i>kelberi</i>	33.01	1.73	7.16	3.57	11.75	9.87
<i>C. piquiti</i>	34.01	1.52	7.16	3.65	11.35	9.80
Hybrid	36.23	1.78	7.72	3.86	12.58	10.79

When these measurements were size-standardized and converted into ecomorphological indices, hybrids consistently showed intermediate values relative to the two parental species, with the exception of relative caudal peduncle length (Supplementary Figure 1). The ecomorphological indices revealed significant

differences between *Cichla ocellaris* var. *kelberi* and *C. piquiti* for some of the analyzed variables (Table 2). Relative eye width differed significantly between the species ($F(2,41) = 3.41$; $p = 0.0427$), with *C. ocellaris* var. *kelberi* showing higher values. Body depth index also showed a significant difference ($F(2,41) = 3.43$; $p = 0.0421$), being higher in *C. ocellaris* var. *kelberi*. These results indicate that *C. ocellaris* var. *kelberi* tends to have proportionally larger eyes and a deeper body compared to *C. piquiti*.

When these measurements were size-standardized and converted into ecomorphological indices, hybrids consistently showed intermediate values relative to the two parental species, with the exception of relative caudal peduncle length (Supplementary Figure 1). One-way ANOVA revealed significant differences for relative eye width ($F(2,41) = 3.41$; $p = 0.0427$) and body depth index ($F(2,41) = 3.43$; $p = 0.0421$). However, Tukey's HSD post-hoc test only confirmed significant differences between the parental species for relative eye width ($p = 0.037$), with *Cichla ocellaris* var. *kelberi* exhibiting higher mean values. Regarding the body depth index, although the ANOVA was significant, the post-hoc comparison between *C. ocellaris* var. *kelberi* and *C. piquiti* revealed only a marginal trend ($p = 0.075$). These results indicate that *C. ocellaris* var. *kelberi* tends to have proportionally larger eyes and suggest a trend toward a deeper body compared to *C. piquiti*.

Table 2. Results of the ecomorphological indices comparing *Cichla ocellaris* var. *kelberi*, *C. piquiti* and hybrids, including F-values, p-values, significance, and species with higher mean values for each variable.

Variable	F(2,41)	p-value	Significant differences	Higher mean value
Relative eye width	3.41	0.0427	<i>kelberi</i> $\neq$ <i>piquiti</i>	<i>C. ocellaris</i> var. <i>kelberi</i>
Relative head length	0.58	0.565	—	—
Relative head depth	1.01	0.372	—	—
Relative caudal peduncle depth	3.14	0.0537	—	—
Body depth index	3.43	0.0421	—	<i>C. ocellaris</i> var. <i>kelberi</i>

Assessment of measurement error

The ME ANOVA (Supplementary Table 2) revealed that individual variation accounted for the largest proportion of the total variation (96.4%), while measurement error represented a small but statistically significant portion. Systematic ME (i.e., bias) accounted for only 2.8% of the total variation, with a significant signal-to-noise ratio (SNR = 0.087, $p = 0.008991$), indicating a minor but consistent directional bias in landmark placement. Random ME contributed 3.3% of the total variation, suggesting that replicates were generally consistent.

Shape variation and differentiation among groups

The wireframe plot (Figure 2) illustrates the mean shape difference between *C. ocellaris* var. *kelberi*, *C. piquiti*, and hybrids following GPA. Distinct shape differences were observed between the two parental species, with *C. ocellaris* var. *kelberi* exhibiting a broader head, deeper body profile and narrower caudal region, whereas *C. piquiti* presented a narrower head and body, and relatively broader caudal region. In addition, *C. piquiti* showed eyes with a smaller diameter compared to *C. ocellaris* var. *kelberi*. Hybrids presented an intermediate shape between both parental species, but more similar to *C. ocellaris* var. *kelberi*, likely due to most hybrids having a greater *C. ocellaris* var. *kelberi* genetic ancestry (Table S1).

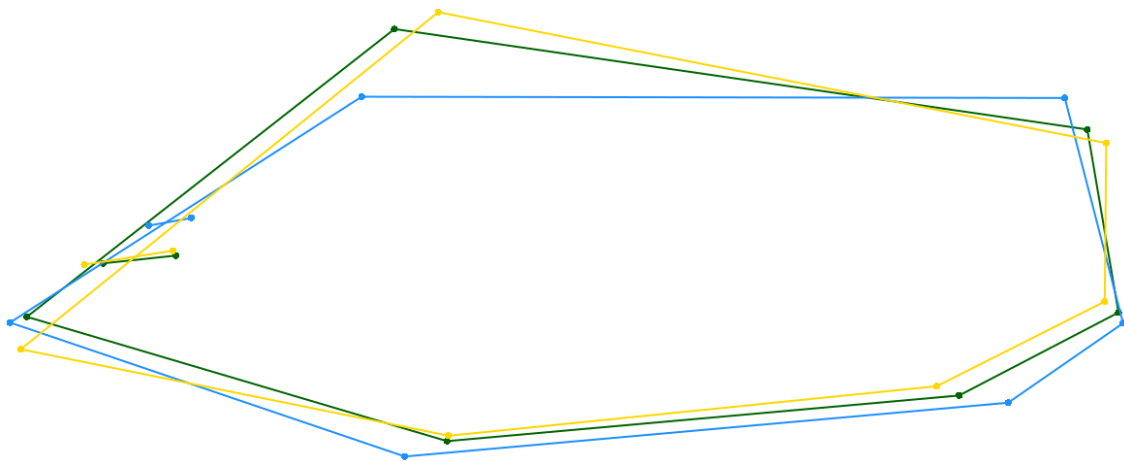


Figure 2. Mean shape configurations of the groups obtained after Generalized Procrustes Analysis (GPA) (yellow = *C. ocellaris* var. *kelberi*, blue = *C. piquiti*, green = hybrids).

The Procrustes ANOVA revealed significant differences in body shape among the three groups (*Cichla ocellaris* var. *kelberi*, *C. piquiti* and hybrids) (Table 4). The effect of group was statistically significant ($F = 3.398$, $Z = 3.806$, $p = 0.001$), accounting

for 14.2% of the total shape variation. Post hoc pairwise comparisons (Table 5) showed that shape differences between *C. piquiti* and *C. ocellaris* var. *kelberi* were highly significant (distance = 0.02924, $p = 0.001$), indicating strong morphological divergence between the two parental species. Hybrids were significantly different from *C. piquiti* (distance = 0.02107, $p = 0.003$), but not from *C. ocellaris* var. *kelberi* (distance = 0.01152, $p = 0.482$), supporting the visual assessment that the average hybrid shape is more similar to *C. ocellaris* var. *kelberi*.

Table 4. Summary of the Procrustes ANOVA testing for significant differences in mean body shape among the three groups (*Cichla ocellaris* var. *kelberi*, *C. piquiti*, and hybrids).

Source	Df	SS	MS	Rsqr	F	Z	Pr(>F)
Groups	2	0.00645	0.003230	0.14218	3.398	3.806	0.001 ***
Residuals	41	0.03897	0.000951	0.85782			
Total	43	0.04543					

Table 5. Results of the post-hoc pairwise comparisons following the significant Procrustes ANOVA

Group Comparison	Distance (d)	UCL (95%)	Z-score	p-value
Hybrid vs. <i>C. ocellaris</i> var. <i>kelberi</i>	0.01152	0.01673	0.483	0.482
Hybrid vs. <i>C. piquiti</i>	0.02107	0.01641	2.885	0.003 **
<i>C. piquiti</i> vs. <i>C. ocellaris</i> var. <i>kelberi</i>	0.02924	0.01809	3.930	0.001 ***

Morphospace Occupation and Morphogenetic Correlation

Principal Component Analysis (PCA) of body shape revealed that the first two principal components (PCs) accounted for 48.84% of the total shape variation (PC1 = 27.28%; PC2 = 21.56%) (Figure 3). The CVA provided a clearer visualization of group separation, with CV1 effectively discriminating between the pure parental species (Supplementary Figure 2).

To formally test the relationship between body shape and genetic ancestry, a Pearson correlation was performed between the scores from the first canonical variate

(CV1) and the Hybrid Index (HI) derived from SNP data. The analysis revealed a strong and significant correlation ($r = -0.893$, $P < 0.001$; Figure 4). This result demonstrates that an individual's position along the primary axis of morphological differentiation is a direct and predictable reflection of its genomic ancestry. Individuals with low HI values (ancestrally *C. ocellaris* var. *kelberi*) clustered at one end of the CV1 axis, while individuals with high HI values (ancestrally *C. piquiti*) clustered at the opposite end. Hybrids formed a continuous gradient between these two parental poles.

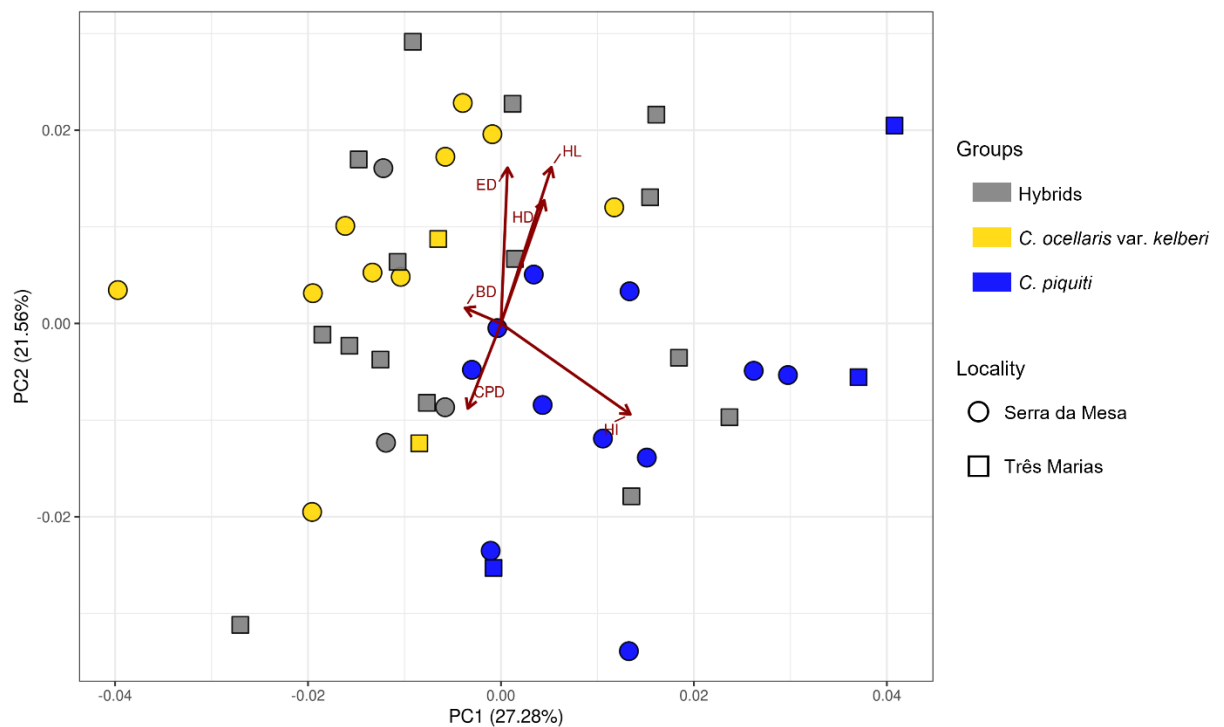


Figure 3. Principal Component Analysis (PCA) of body shape variation derived from geometric morphometrics. Points represent individual specimens, colored by their assigned group. The red vectors illustrate the correlation of six supplementary variables: Hybrid Index (HI – proportion of *C. piquiti* ancestry), body depth (BD), eye diameter (ED), head depth (HD), head length (HL) and caudal peduncle depth (CPD).

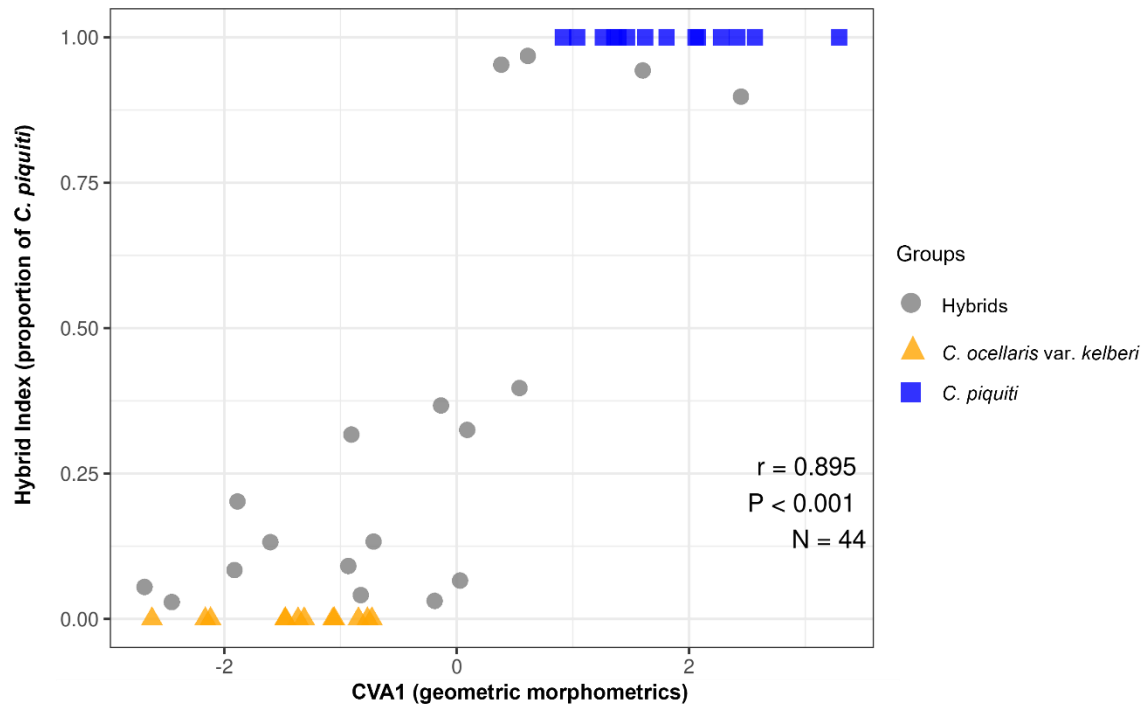


Figure 4. Correlation between the primary axis of morphological shape differentiation (CV1) and the genomic Hybrid Index (HI). Points are colored by their a priori group assignment: *C. ocellaris* var. *kelberi* (yellow), *C. piquiti* (blue), and hybrids (grey).

Morphological disparity

An analysis of morphological disparity was conducted to test whether hybridization led to an increase in phenotypic variation. The results showed no significant differences in the amount of shape variation among the groups ($P > 0.05$ for all pairwise comparisons) (Table 6). The Procrustes variance for the hybrid group was not significantly different from that of *C. ocellaris* var. *kelberi* ($P = 0.846$) or *C. piquiti* ($P = 0.493$). This indicates that while hybrids are morphologically intermediate, they do not exhibit a greater degree of shape variation than either parental species.

Table 6. Morphological disparity analysis among genetic groups. Values on the diagonal (in bold) represent the Procrustes Variance for each group. Values in the lower triangle represent the P-values from pairwise comparisons based on 999 permutations.

	<i>C. ocellaris</i> var. <i>kelberi</i>	<i>C. piquiti</i>	Hybrids
<i>C. ocellaris</i> var. <i>kelberi</i>	0.000900	—	—
<i>C. piquiti</i>	0.665	0.000805	—
Hybrids	0.846	0.493	0.000939

Discussion

Our results provide a quantitative characterization of body shape variation in *C. ocellaris* var. *kelberi* and *C. piquiti* and the morphological consequences of hybridization between them. The primary finding is that the two parental species possess contrasting ecomorphological shapes based on geometric morphometrics data. *Cichla ocellaris* var. *kelberi* is defined by a more robust, deep-bodied form with a broader head, whereas *C. piquiti* has a more streamlined, slender shape with a narrower head and a broader caudal region. Furthermore, the analysis shows that hybrid individuals exhibit a body shape that is mostly intermediate to the two parental species. This intermediacy was demonstrated by a strong and significant correlation ($r = -0.893$, $P < 0.001$) between the main axis of morphological differentiation from the Canonical Variate Analysis (CV1) and the genomic Hybrid Index (HI). This strong association indicates that an individual's body shape is a reliable predictor of its genomic ancestry. Complementary ecomorphological analyses based on linear measurements supported the geometric morphometric results, reinforcing the distinction between the two parental species and the intermediate morphology of hybrids. Our results were congruent with the traditional morphometric description of the species by Kullander & Ferreira (2006), where *C. ocellaris* var. *kelberi* displayed higher mean values of head length, head depth, body depth and orbital diameter.

The morphological divergence between the two parental species likely reflects adaptations for niche partitioning in their native sympatric range, consistent with the general pattern observed among sympatric *Cichla* species, which typically occupy

distinct habitats and exploit different resources (Jepsen et al. 1997; Willis et al. 2012). *Cichla ocellaris* var. *kelberi* usually occupies littoral areas of the river which is more structurally complex (vegetation), while *C. piquiti* is associated with deeper, less structured mid-channel habitats (Winemiller et al. 2021). In habitats with high structural complexity, light levels are often low due to both depth-related attenuation and shading from aquatic vegetation or submerged debris (Lythgoe, 1979; Lisney et al. 2020). In response, fish from complex habitats develop eyes with larger diameter (Dobberfuhl et al. 2005; Lisney et al. 2020). Consistent with the hypothesis of adaptation to structurally complex, low-light littoral habitats, *C. ocellaris* var. *kelberi* showed a significantly larger relative eye width than *C. piquiti*, suggesting selection for enhanced visual sensitivity in shaded environments. Deeper bodies are also frequently associated with maneuverability in structurally-complex environments (Feilich 2016). In contrast a more streamlined, fusiform body is an adaptation that minimizes hydrodynamic drag, thereby enhancing efficiency for sustained swimming (Webb 1984). *C. ocellaris* var. *kelberi* exhibited a significantly greater Body Depth Index, reinforcing its adaptation for enhanced maneuverability within complex littoral zones. The distinct ecomorphologies of the parental species, therefore, represent a classic functional trade-off between maneuverability in structurally complex habitats and cruising efficiency in open water, a common pattern of niche partitioning in riverine fish assemblages (Gatz, 1979). However, despite previous evidence suggesting habitat segregation between these species in their native environments, recent ecological observations by Andrade and Pelicice (2022) showed that *C. ocellaris* var. *kelberi* and *C. piquiti* can coexist within the same reservoir with a high degree of habitat overlap. Further studies are needed to determine whether this pattern results from the altered environmental conditions of reservoirs or represents the typical interaction between these species.

The intermediate morphology of the hybrids may confer an intermediate functional performance. Selection against intermediate phenotypes may occur when intermediate environments are uncommon (Redenbach and Thaylor 2003; Bossu and Near 2013; Arnegard et al. 2014). Such divergent selection could help explain why hybrids between these two species are rare in their native range. Willis et al. (2012) argued that the ability of hybrids between relatively divergent *Cichla* species to reproduce suggests an important role for pre-zygotic barriers in this genus. However, the occurrence of reproduction in hybrids does not exclude the existence of post-zygotic barriers, because hybrids with reduced fitness may still reproduce at low rates. Our

results are consistent with this possibility, since all hybrids detected in the native area were late-generation hybrids, and backcrosses typically exhibit fitness levels comparable to those of the parental species. However, because phenotypic fitness is context-dependent, the divergent selection that may act against hybrids in native habitats could be absent or even reversed in novel or disturbed ecosystems (Hatfield & Schluter 1999; Thompson et al. 2022). In such environments, intermediate phenotypes could allow them to exploit a broader range of resources or thrive in intermediate habitats where neither parental species excel (Seehausen 2004). Notably, our analysis of morphological disparity revealed that this intermediacy is not accompanied by an increase in phenotypic variance. The Procrustes variance within the hybrid group was statistically equivalent to that of the parental species, and hybrid morphology was intermediate between them, consistent with expectations under predominantly additive genetic effects on shape. The evolutionary potential of this hybrid swarm, therefore, seems to stem not from the generation of radical new forms, but from the ecological success of the intermediate phenotypes.

In the Serra da Mesa (SM) reservoir, where parental forms are predominant and hybrids are rare, the prevailing ecological conditions may continue to exert disruptive selection against intermediate phenotypes. Conversely, the dominance of hybrids in the Três Marias (TM) reservoir strongly suggests that the selective pressures that reduce hybrid fitness in other contexts are relaxed. This pattern suggests that the specific ecological conditions within the TM reservoir, whether related to introduction history, resource availability, or physical habitat structure, allowed hybrids to proliferate and establish a stable hybrid swarm (Taylor et al., 2006; Seehausen, 2004). This is not unique to our study sites but reflects a broader, well-documented pattern for these *Cichla* species. Within their native range, even in large impoundments, species boundaries between *C. ocellaris* var. *kelberi* and *C. piquiti* are typically maintained, resulting in little to no levels of natural hybridization (Willis et al. 2012; Carvalho et al. 2014). However, when these species are co-introduced outside their native range, it often results in widespread introgression and the formation of extensive hybrid swarms (Oliveira et al. 2006; Almeida-Ferreira et al. 2011; Nóbrega et al. 2023).

While there is a strong correlational link between genotype, phenotype, and ecological context, the functional mechanisms underlying the success of hybrid phenotypes remain to be directly tested. Experimental studies assessing swimming performance, feeding efficiency, or habitat preference could help determine whether

intermediate morphologies confer functional advantages or trade-offs under different environmental contexts. Furthermore, ecological studies are needed to confirm the presumed niche use. Diet analyses could reveal if hybrids occupy an intermediate trophic position or exploit a broader range of resources. Answering these questions will be vital to move from a descriptive and correlational understanding to a mechanistic one, fully elucidating the drivers and consequences of successful hybrid populations. This study highlights the integration of genomic and morphometric data to understand complex evolutionary and ecological phenomena. The use of SNP data from a previous study was essential, as it provided the a priori group definitions (*C. ocellaris* var. *kelberi*, *C. piquiti*, and hybrids) necessary to frame all subsequent morphological analyses. Without this genomic foundation, the continuum of morphological variation would have been difficult, if not impossible, to interpret. In turn, geometric morphometrics provided the crucial phenotypic context, revealing how genomic admixture translates into a tangible gradient of body shapes.

Conclusion

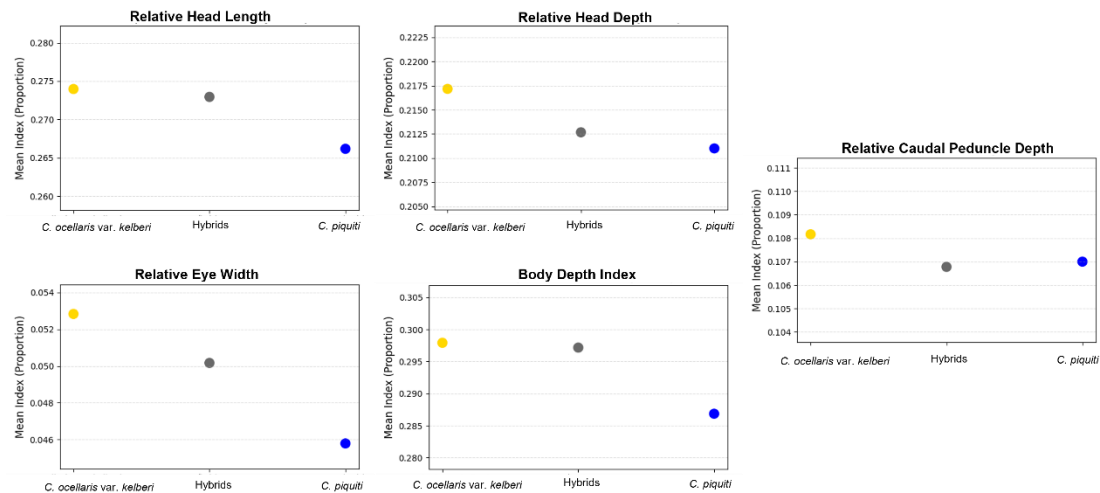
Our results provide a morphometric characterization of hybridization between *Cichla ocellaris* var. *kelberi* and *C. piquiti*, demonstrating that the parental species possess distinct ecomorphological shapes. We also demonstrated that hybrids span a continuous morphological gradient between the parental phenotypes, with individual morphology closely tracking genomic ancestry. The success of this hybrid swarm does not appear to be driven by the generation of novel, transgressive forms, as morphological disparity was not increased in the hybrid group. These findings reinforce the predictive power of geometric morphometrics for identifying hybrid individuals and understanding the phenotypic outcomes of admixture. This study illustrates how the integration of genomic and morphological data can reveal the ecological and evolutionary dynamics of hybridization.

Acknowledgements

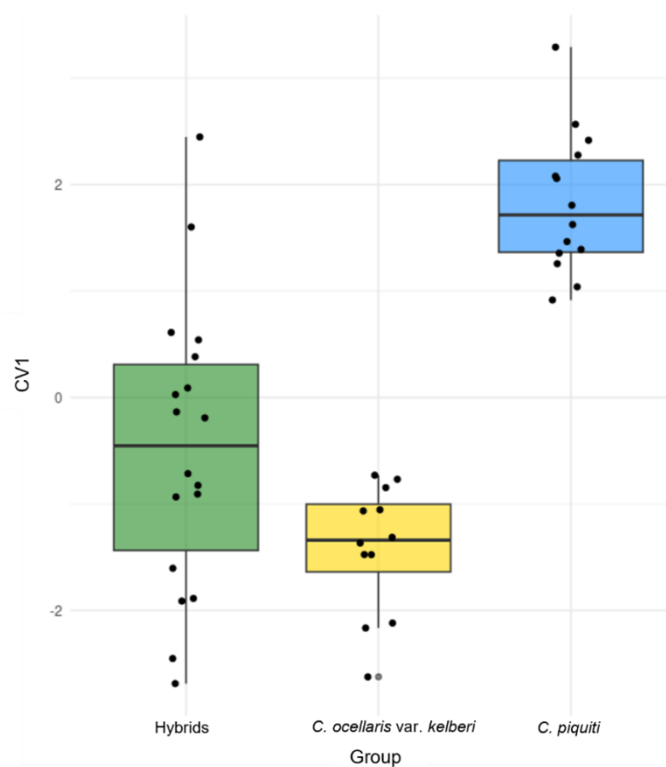
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Supplementary Figures



Supplementary Figure 1. Boxplot of ecomorphological indices for *C. ocellaris* var. *kelberi* (yellow), hybrids (gray), and *C. piquiti* (blue).



Supplementary Figure 2. Distribution of CV1 scores from the Canonical Variate Analysis for *C. ocellaris* var. *kelberi*, *C. piquiti*, and hybrids.

Supplementary Tables

Supplementary Table 1. Hybrid index (HI) values representing the proportion of *C. piquiti* ancestry for all sampled individuals from Serra da Mesa (SM) and Três Marias (TM). Values close to 0 indicate *C. ocellaris* var. *kelberi* ancestry, values close to 1 indicate *C. piquiti* ancestry, and intermediate values reflect varying degrees of admixture.

Sample	HI (proportion of <i>C. piquiti</i>)
SM12	0.000010
SM13	0.000010
SM18	0.000010
SM30	0.000010
SM33	0.000010
SM35	0.000010
SM37	0.000010
SM38	0.000010
SM5	0.000010
SM7	0.000010
SM36	0.031631
SM34	0.041742
SM31	0.968267
SM1	0.999990
SM19	0.999990
SM2	0.999990
SM24	0.999990
SM25	0.999990
SM27	0.999990
SM29	0.999990
SM3	0.999990
SM32	0.999990
SM6	0.999990
SM8	0.999990
TM10	0.000010

TM28	0.000010
TM18	0.029448
TM16	0.055689
TM19	0.066426
TM15	0.084808
TM26	0.091892
TM14	0.132429
TM12	0.133315
TM1	0.202521
TM7	0.317246
TM20	0.325232
TM13	0.367359
TM23	0.397727
TM25	0.898779
TM6	0.943626
TM31	0.953805
TM11	0.999990
TM21	0.999990
TM3	0.999990

Supplementary Table 2. Summary of the Measurement Error ANOVA (ME ANOVA) used to assess the contribution of biological variation (Subjects) versus systematic and random measurement error to the total shape variance. Significance for the Signal-to-Noise Ratios (SNR) was determined using 999 permutations.

Source	Df	SS	MS	Rsqr	EtaSq.ME	SNR	Z	Pr(>SNR)
Subjects	43	0.0867	0.002	0.964	—	29.492	16.735	0.000999
Systematic ME	1	0.0002	0.0002	0.002	0.080	0.087	2.406	0.008991
Random ME	43	0.0029	0.00006	0.032	0.920	—	—	—
Total	87	0.0899	—	—	—	—	—	—

Considerações Finais

A presente tese estruturou-se sob uma abordagem integrativa para investigar a hibridização e a introgressão entre duas espécies de um importante grupo de ciclídeos neotropicais, *Cichla ocellaris* var. *kelberi* e *Cichla piquiti*, comparando suas dinâmicas em ambientes onde coexistem historicamente (nativo) e onde foram introduzidas (não-nativo). Ao unir a revisão sistemática da literatura, a genômica de populações via ddRAD-seq e a morfometria geométrica, pudemos avançar na compreensão dos fatores relacionados à e consequências da hibridização entre essas espécies de tucunaré.

A revisão bibliográfica realizada no primeiro capítulo revelou que a hibridização no gênero *Cichla* não é um evento isolado ou exclusivo de ambientes introduzidos, mas sim um fenômeno geograficamente e filogeneticamente disseminado em sua área nativa. Identificamos que a hibridização ocorre em zonas de contato naturais, sugerindo que o fluxo gênico desempenhou, e continua desempenhando, um papel relevante na história evolutiva do grupo. Em ambientes não-nativos, o padrão se altera drasticamente, de forma que a frequência de híbridos aumenta exponencialmente, muitas vezes superando em números as espécies parentais.

No segundo capítulo, a aplicação de marcadores genômicos de alta resolução (SNPs) proporcionou uma visão inédita sobre a dinâmica de hibridização em populações nativas e introduzidas de *C. ocellaris* var. *kelberi* e *C. piquiti*. Essa abordagem foi fundamental para aplicar testes estatísticos robustos (como o D3 e bgc), permitindo identificação de introgressão na área nativa e caracterização de introgressão diferencial nas populações introduzidas. A detecção de introgressão na área nativa levanta o questionamento acerca de se a hibridização nesse contexto ocorre de forma difusa ou é limitada a zonas híbridas específicas. Trabalhos futuros com amostragens mais amplas, tanto em ambientes modificados (reservatórios), quanto em áreas naturais, poderão melhor elucidar essa questão. Em contrapartida, no ambiente invadido (Três Marias), confirmou-se a formação de um enxame híbrido com retrocruzamentos avançados. A alta heterozigosidade encontrada nesses híbridos sugere que a introgressão pode estar atuando como um mecanismo de "resgate genético", mitigando efeitos fundadores e potencialmente aumentando o potencial adaptativo e a invasividade destas populações. A assimetria detectada na introgressão, favorecendo o genoma de *C. ocellaris* var. *kelberi* mas retendo alelos específicos de *C. piquiti*, aponta para uma dinâmica seletiva, onde a hibridização pode conferir vantagens adaptativas

no novo ambiente. Análises de outras populações híbridas introduzidas poderão revelar se introgressão de alelos de *C. piquiti* no genoma de *C. ocellaris* var. *kelberi* é uma regra geral para a coexistência dessas espécies nesses ambientes ou esse padrão varia de acordo com o local.

No terceiro capítulo demonstramos que *C. piquiti* e *C. ocellaris* var. *kelberi* possuem ecomorfologias distintas, o primeiro mais hidrodinâmico e adaptado a águas abertas, e o segundo com corpo mais alto e manobrável, e olhos maiores, típico de ambientes estruturados. A hibridização resultou em um gradiente morfológico intermediário, com uma correlação forte entre a forma do corpo e o índice híbrido genômico. A ausência de transgressão morfológica sugere que os híbridos não estão explorando nichos fora do alcance adaptativo das espécies parentais, mas possuem a capacidade de ocupar um espaço funcional intermediário. Estudos sobre a dieta e reprodução de indivíduos híbridos poderá elucidar o real nicho ocupado por esses híbridos e sua posição em relação às espécies parentais. O fenótipo intermediário dos híbridos sugere que, em seus ambientes nativos, a seleção disruptiva atua como uma barreira pós-zigótica. A persistência desses fenótipos intermediários nos ambientes invadidos sugere que essa seleção está relaxada ou ausente nesses contextos.

Estes resultados possuem implicações diretas para a conservação e manejo de espécies de tucunaré. Na conservação de estoques nativos, a detecção de introgressão críptica em reservatórios pode ser usada para monitorar a integridade genética desses estoques, orientando estratégias de manejo para minimizar os impactos dos represamentos. No contexto do manejo de espécies invasoras, a elevada diversidade genética e a prevalência de híbridos observadas neste estudo sugerem um considerável potencial evolutivo nessas populações e levantam a possibilidade de que indivíduos híbridos possam contribuir para o estabelecimento de novas populações invasoras caso ocorram eventos de translocação.

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