

# Inter- and intra-basin phenotypic variation in two riverine cichlids from northeastern Brazil: potential eco-evolutionary damages of São Francisco interbasin water transfer

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**Abstract** Intraspecific morphological variation can be generated by a set of historical and ecological processes, and can be induced by anthropogenic actions. One such activity that has large-scale environmental impacts in freshwater environments is interbasin water transfer. Brazil's Mid-Northeastern Caatinga freshwater ecoregion is going through an interbasin diversion that will link waters from São Francisco ecoregion with the four major basins of Mid-Northeastern Caatinga.

Here, we aimed to evaluate the morphological variation of two cichlids, *Cichlasoma orientale* and *Crenicichla menezesi*, from both ecoregions and test, whether this morphological variation is related to historical isolation among basins and/or to local water flow regimes, through mtDNA (cytb) and geometric morphometric approaches. Our results showed, for both species, significant morphological differences among basins. However, the comparisons between genetic and morphological differences among basins were not significant. Additionally, a significant shared morphological pattern between species (body depth and mouth position) among different habitats was found. These results reinforce a potential role of environmental pressures upon fish morphology, possibly through phenotypic plasticity. The evolutionary role of morphological responses in freshwater fish populations—especially in variable environments—is discussed, as well as the potential eco-evolutionary damages that might result from the artificial river connection.

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## Introduction

Phenotypes are the interface of eco-evolutionary dynamics. Indeed, selection acts directly on phenotypes, whereas it acts only indirectly on genotypes

through selection upon the phenotypes that it generates. Thus, a full understanding of eco-evolutionary dynamics requires the study of phenotypic variation (Hendry, 2013). Diversification of traits can occur rapidly (e.g. sexual selection or local adaptations) or slowly (e.g. geographic isolation and its associated genetic drift), but the influences of these processes on neutral genetic variation may be markedly different (Mullen et al., 2009). Rapid evolution of phenotypic traits can obscure understanding of the phylogenetic relationships among populations because insufficient time may have passed for the accumulation of genetic differences at markers not associated with the traits under diversifying selection (i.e., incomplete lineage sorting) (Hull et al., 2010). However, if phenotypic differences are attributable to long-term isolation and subsequent genetic drift, genetic differences would presumably be ubiquitous across evolutionarily neutral loci (White et al., 2013).

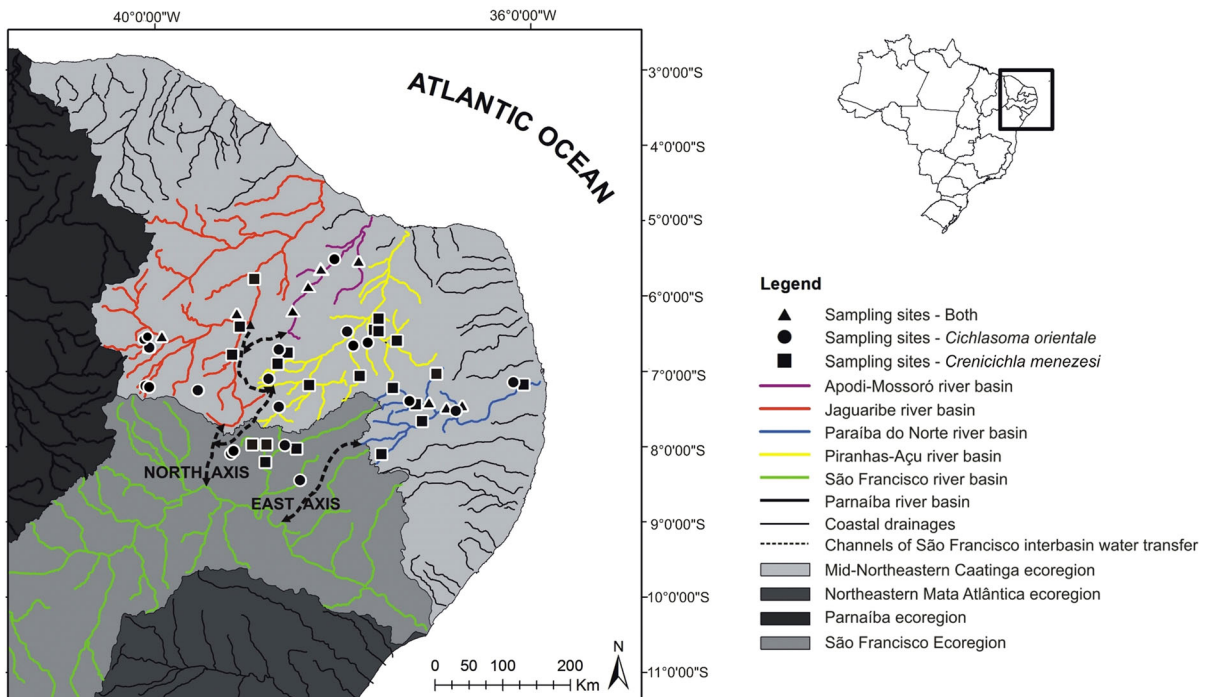
Natural populations are often exposed to variable environmental conditions, and must adapt to these conditions (Windig et al., 2004). While natural changes have always produced such variation, anthropogenic effects have been altering the natural environment at an unprecedented rate (Reed et al., 2011). Freshwater environments have been particularly impacted by anthropogenic actions and represent one of the most rapidly declining habitats on Earth (Collen et al., 2013). Projects of interbasin water transfer, in particular, stand out due to their large scale, high costs, and serious environmental impacts upon freshwater habitats (Meador, 1992; Pittock et al., 2009; Grant et al., 2012). Despite their usefulness for generating water supply, the artificial links between waterways that are generated by these projects may function as connections for aquatic biota (Moreira-Filho & Buckup, 2005; Lima & Caires, 2011). Such connectivity between previously isolated basins could lead to changes in aquatic diversity, including biodiversity homogenization, loss of species richness (Bellafronte et al., 2010; Grant et al., 2012), and modifications in gene flow patterns (Blanco et al., 2009; Peres et al., 2012).

Furthermore, interbasin water transfer works have large ecological damaging effects on freshwater habitats. Disturbances on water chemistry and quality, alteration of habitats, and introduction of invasive species are some of the potential damages reported for interbasin water transfer works (Meador, 1992).

Changes on the natural water flow regime, which is considered to be one of the most influential variables with regard to morphological diversification in aquatic organisms including fishes (Langerhans, 2008), crustaceans (Zimmerman et al., 2011), and tadpoles (Haad et al., 2011), are also reported as potential effects of interbasin water transfers (Meador, 1992).

Northeastern Brazil is classified as one of the most unknown ichthyological regions of the world (Rosa et al., 2003; Lévêque et al., 2008), and is highly threatened by anthropogenic activities (Collen et al., 2013). Efforts to manage and conserve the natural water flow regimes of this region have been hampered by the scarcity of scientific information regarding the ecological processes associated with its biota (Malthick & Medeiros, 2006). This political region comprises four freshwater ecoregions, divided mainly by biogeographical barriers: Parnaíba, São Francisco, Northeastern Mata Atlântica (southwards to São Francisco river basin), and Mid-Northeastern Caatinga (between São Francisco and Parnaíba basins) (Abell et al., 2008; Albert & Reis, 2011) (Fig. 1). Mid-Northeastern Caatinga is predominantly in the semi-arid Caatinga biome, which is characterized by its dry weather and impermeable soil, resulting in an intermittent and seasonal river flow regime (Rosa et al., 2003; Rocha et al., 2012). Due to the constant regimes of droughts, limited water supply has historically been a serious issue for humans living in this region (Araújo et al., 2004). In the past, numerous efforts from the government have unsuccessfully tried to solve the insufficient water supply in the Mid-Northeastern Caatinga region (Pittock et al., 2009). A large governmental interbasin water transfer project was approved in 2006 and is currently underway. This work will pump waters from the São Francisco river basin (SFR) to the four major basins of the Mid-Northeastern Caatinga ecoregion. Two main axes are proposed, summing a total of 720 km, with water capture at two adjacent sites of the main channel of the Sub-Middle São Francisco river basin. The north axis will link São Francisco waters with the Jaguaribe (JAG), Piranhas-Açu (PAC), and Apodi-Mossoró (APO) river basins, and the east axis will link São Francisco waters with the Paraíba do Norte (PAR) river basin (Pittock et al., 2009) (Fig. 1).

Several processes can have effects on freshwater fishes morphological variation, including historical isolation (Jones & Johnson, 2009), environmental



**Fig. 1** Map of sampling locations for both species. Dashed line indicates the two major canals of São Francisco interbasin water transfer project

conditions (Langerhans et al., 2003; Langerhans, 2008; Crispo & Chapman, 2010) and anthropogenic effects (Haas et al., 2010; Franssen et al., 2012; van Rijssel & Witte, 2012). Cichlidae is particularly well suited for such studies because it is a species-rich group that exhibits remarkable morphological, ecological, and behavioral diversity (Kocher, 2004). Inter- and intraspecific morphological variation among cichlids has already been linked to historical (Ready et al., 2006; Fan et al., 2012) and/or ecological processes (Langerhans et al., 2003; Trapani, 2003; Stelkens & Seehausen, 2009; Crispo & Chapman, 2010, 2011), but little is known about the evolutionary processes in Neotropical cichlids (Fan et al., 2012; López-Fernández et al., 2013).

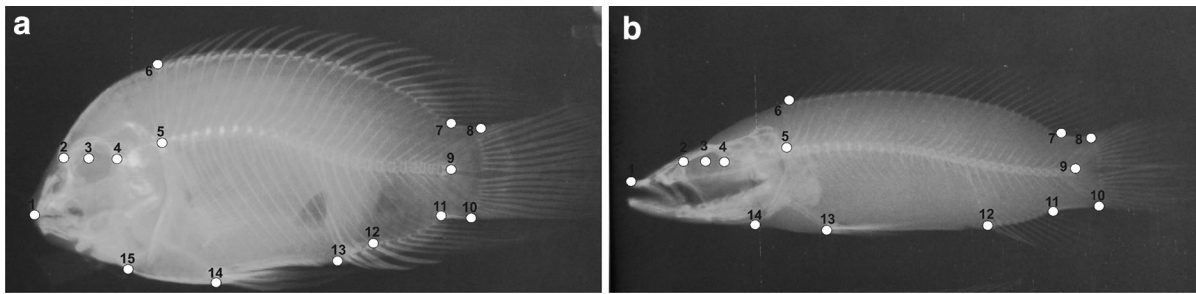
The responses of aquatic biota to seasonally variable habitats, which typify rivers and streams in the semi-arid region of Brazil, are poorly known (Maltchik & Medeiros, 2006). Nonetheless, the potential loss of morphological variation among and within populations would contribute to a loss of diversity, signifying serious evolutionary damage in an already threatened regions such as northeastern Brazil. In this context, we aimed to analyze the

morphological variation of two riverine cichlids (*Cichlasoma orientale* Kullander 1983 and *Crenicichla menezesi* Ploeg 1991) from semi-arid northeastern Brazil and evaluate whether the morphological variation is related to a historical isolation among basins (and its evolutionary consequences) and/or local water flow conditions. Morphological, molecular, and ecological data will be used to answer these major questions. Furthermore, we will discuss some of the potential impacts of the ongoing São Francisco interbasin water transfer on evolutionary trends in freshwater fish populations.

## Materials and methods

### Study taxa and sampling

*Cichlasoma orientale* and *C. menezesi* are two cichlid fishes that are widely distributed among isolated basins on Northeastern Brazil, including those that will be affected by the São Francisco interbasin water transfer project (Kullander, 1983; Ploeg, 1991; Rosa et al., 2003).



**Fig. 2** X-ray photo of **a** *Cichlasoma orientale* and **b** *Crenicichla menezesi*. White circles indicate the landmarks used in geometric for analysis of shape morphology. Location and numbering are: 1 tip of the snout, 2 anterior margin of the eye, 3 center of eye, 4 posterior margin of the eye, 5 anterior insertion of column, 6 anterior insertion of dorsal fin, 7 posterior insertion

of dorsal fin, 8 dorsal insertion of caudal fin, 9 posterior insertion of spine, 10 ventral insertion of caudal fin, 11 posterior insertion of anal fin, 12 anterior insertion of anal fin, 13 anterior insertion of pelvic fin, 14 ventral opening of the operculum, 15 insertion of third spine of the anal fin (only for *C. orientale*)

Sampling was conducted between March 2012 and August 2014, using hand-nets, line and hooks, trawls, and gill nets. The fishes were anesthetized using clove oil and followed the current proper animal care protocols. Specimens were individually labeled and tissue (muscle or fin clip) samples were preserved in ethanol 95%. Afterwards, the individuals were fixed in 10% formalin and then stored in 70% ethanol. Voucher specimens were deposited in the ichthyological collections of Universidade Federal da Paraíba (UFPB) and Universidade Federal do Rio Grande do Norte (UFRN) (Table S1).

Sampling locations were classified according to the predominant water flow regime, following the classification proposed by Matthews (1998). In general, lentic habitats were defined as a water bodies with still water, such as reservoirs, lakes, and artificial pounds. Lotic habitat were defined as water bodies with downstream water flow, such as rivers, streams, and waterfalls (Table S1).

### Morphological variation

To evaluate morphological variation, geometric morphometrics methods were performed. Recent studies have demonstrated a significant influence of preservation methods (formalin, ethanol, or freezing) on shape analysis using geometric morphometrics (Berbel-Filho et al., 2013; Martinez et al., 2013). To avoid this potential confounding artifact, we chose X-ray plates as an alternative method for morphological data collection. With each individual placed in the same orientation, X-ray images were captured using an

Intecal<sup>®</sup> 300 machine and developed with a Fuji<sup>®</sup> FPM100A desktop processor. Body depth (vertical measure of pelvic fin insertion to dorsal fin insertion) was measured manually with a 300 mm digital caliper to be used as a size scale for each specimen image. Only adult individuals were included on morphological analysis.

Using TPSdig version 1.4 software (Rohlf, 2004), 15 two-dimensional landmarks were taken from 187 *C. orientale* adult specimens (114.8–70.4 mm SL) (Fig. 2a), and 14 landmarks were taken from 135 *C. menezesi* adult specimens (109.3–65.7 mm SL) (Fig. 2b). An additional landmark from *C. orientale* (Landmark 12, Fig. 2a) was assigned on the insertion of the anal fin's fourth spine, because it is an important taxonomic trait in *Cichlasoma* species (Kullander, 1983). Alignment of the landmarks was done using the Procrustes superposition method in MorphoJ software version 1.05 (Klingenberg 2011). To account for allometric effects on shape variation, we performed a multivariate regression of Procrustes coordinates (shape), using the log of centroid size; we used the residuals of this regression analysis as data for the subsequent analysis, as recommended by Klingenberg & Marugan-Lobón (2013). The sexual dimorphism reported for *C. orientale* is related to size (Gurgel et al., 2011) and for *C. menezesi* to color patterns during reproductive stage (Araújo et al., 2012). As we are evaluating shape variation, we pooled the sexes for morphological analyses.

To evaluate the relative contribution of the actual basin isolation and habitats (lotic and lentic) to morphological variation, a Procrustes ANOVA was

conducted (Klingenberg & McIntyre, 1998) using the “geomorph” package (Adams & Otárola-Castillo, 2013). To determine whether the effects of basin isolation and habitat were statistically significant, we calculated  $F$  statistics (Goodall, 1991) with 1000 residual randomization permutation procedures (RRPP) (Collyer et al., 2014). All analyses were done using R software v. 3.1.0 ([www.R-project.org/](http://www.R-project.org/)).

In order to evaluate morphological variation among basin populations, a canonical variable analysis (CVA) was conducted to test the effectiveness of variation in predicting a priori groups (in our case, the river basins) using a  $T^2$  hotelling test (Zelditch et al., 2004). A Mahalanobis pairwise distance matrix was used as morphological distance matrix of the populations. A warped outline drawing graphic was chosen to visualize all the morphological variations. All of these analyses were made in MorphoJ software version 1.05 (Klingenberg, 2011).

Since water flow regime is an influential variable in aquatic environments, we aimed to analyze whether different water flow regimes (lentic-lotic) had an effect on intraspecific morphological variation. To address this, we conducted a discriminant analysis using the individuals of all basins grouped according to the water flow regime of each sampling location. To determine the intensity of the habitat effect, we performed a  $T^2$  statistical test and a permutation test (10,000 permutations). All the analyses were made in MorphoJ software version 1.05.

#### DNA isolation, amplification, and sequencing

To evaluate if the morphological variation was related to the historical genetic structure among populations distributed in five currently isolated basins, 68 individuals of both species (*C. orientale*: 52 of lotic, 16 of lentic habitats; *C. menezesi*: 50 of lotic, 18 of lentic habitats) from all basins involved in the water diversion project were sequenced. Some small size individuals used on molecular were not included on morphological analysis (Table S2). Genomic DNA extraction was performed with DNeasy<sup>TM</sup> Tissue Kit (Qiagen). The mtDNA gene Cytochrome b (cytb) was amplified, using the following primers: FishCytb-F (5' ACCACCGTTCTTATT-CAACTACAAGAAC 3') and TrucCytB-R (5' CCG ACTTCCGGATTACAAGACCG 3') (Sevilla et al., 2007). The PCR reactions (25  $\mu$ l of final volume) were performed using 10–30 ng of DNA template, 0.25 ng/

$\mu$ l of each primer, 12.5  $\mu$ l of 2x Taq Master Mix Vivantis<sup>TM</sup>, and 10.2  $\mu$ l of ultrapure water. The amplifications used an initial cycle at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 50°C for 35 s, 72°C for 70 s, a final extension step at 72°C for 7 min, and then 20° for 2 min. PCR products were examined on a 1.8% agarose gel and then purified using the QIAquick PCR Purification Kit (Qiagen). All sequencing reactions were performed using the Big Dye v3.1 (Applied Biosystems) and screened in ABI PRISM<sup>®</sup> 3500 Genetic Analyzer (Applied Biosystems). All sequences obtained in this study were deposited in GenBank (Table S2).

#### Analysis of mtDNA sequence data

Sequences were edited using Geneious version 6.1 software (<http://www.geneious.com/>), imported into Mega 5.1 (Tamura et al., 2011), and then aligned using ClustalW. These alignments resulted in 950 base pairs for *C. orientale* and 983 base pairs for *C. menezesi*.

Aiming to understand how the genetic structures of these species vary across populations and habitats, a molecular variance analysis (AMOVA) was performed by Arlequin software v. 3.5 (Excoffier & Lischer, 2010) with 1000 permutations, using  $P$  distance model as the distance method. This analysis was done to test two different hypotheses: (i) the hypothesis of partitioned molecular variation of the current spatial distribution among the five different basins or, (ii) the molecular variation is explained by the different habitats (lotic-lentic).

To delineate the relationships within intraspecific lineages, haplotype networks were generated using TCS1.21 (Clement et al., 2000) with a 95% statistical probability that no multiple substitutions had occurred. The sequences from lotic and lentic habitats were also represented on the haplotype network.

A Mantel test was conducted to evaluate the correlation between the Mahalanobis distances of pairwise populations and the matrix of  $P$  distance between populations as a matrix of genetic structure. This test was done using the available “Ecodist” package (Goslee & Urban, 2007) in R software.

## Results

The Procrustes ANOVA analysis revealed a significant effect of basins on morphological variation for



**Table 1** Partitioned analysis of morphological variance (ANOVA), between basins and habitats (lentic-lotic)

<i>Cichlasoma orientale</i>	<i>df</i>	<i>SS. obs</i>	<i>MS</i>	<i>P</i>
<b>(a)</b>				
Basins	4	0.0179	0.0044	0.0001
Habitats	1	0.0077	0.0077	0.0001
Basins × Habitats	4	0.0153	0.0038	0.0001
Total	186	0.2772	0.0014	NA
<i>Crenicichla menezesi</i>	<i>df</i>	<i>SS. obs</i>	<i>MS</i>	<i>P</i>
<b>(b)</b>				
Basins	4	0.0244	0.0061	0.0019
Habitats	1	0.0030	0.0030	0.2447
Basins × Habitats	4	0.0336	0.0084	0.0001
Total	134	0.3284	0.0022	NA

(a) Results for *Cichlasoma orientale*; (b) Results for *Crenicichla menezesi*

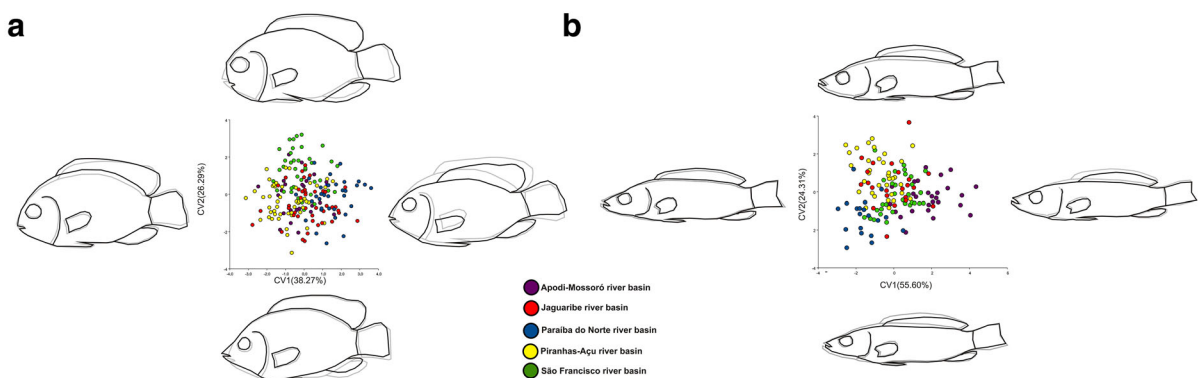
both species. This effect was higher than the habitat effects, which was not significant for *C. menezesi* (basins: *C. orientale*,  $P = 0.0001$ ,  $SS = 0.179$ ; *C. menezesi*,  $P = 0.0021$ ,  $SS = 0.0244$ ; habitats: *C. orientale*,  $P = 0.0001$ ,  $SS = 0.077$ ; *C. menezesi*,  $P = 0.2447$ ,  $SS = 0.030$ ) (Table 1). The interaction between basins and habitats was significant for both species, revealing that the shape variation has different orientations for each factor.

For both species, the CVA showed significant morphological variation among all fish populations (with the exception of Jaguaribe and São Francisco comparison in *C. menezesi*) across basins involved in the transposition project (Table 3). Body depth, mouth

position, snout length, caudal peduncle areas, and length of fins were the main traits that exhibited significant morphological variation (Fig. 3).

In *C. orientale*, the first canonical variable (CV1) explained 38.27% of the shape variation (Fig. 3a). Most of the individuals from São Francisco (28), as well as individuals from the Piranhas-Açu (40), and some from Jaguaribe (12) and Apodi-Mossoró (13) river basins, showed a more elliptical body shape, with deeper heads, decreased snout length, an inferior terminal mouth (sub-terminal mouth), and a larger area of caudal and anal fins. Most of the individuals from Paraíba do Norte (32) and Jaguaribe (30), and some from Apodi-Mossoró (10), Piranhas-Açu (10) and from São Francisco (11) showed a more fusiform shape, with an increased snout length, a more terminal mouth position, a reduced maximum body depth, reduced eye diameter, and a longer caudal fin.

The second Canonical Variable (CV2) explained 26.29% of the total shape variation in *C. orientale*. Most of the individuals from Apodi-Mossoró (12), Paraíba do Norte (17), Piranhas-Açu (34), and Jaguaribe (29) showed a relatively upturned mouth and larger snout, a shallow caudal peduncle, a decrease eye diameter. Some individuals from Apodi-Mossoró (11), Jaguaribe (13), Piranhas-Açu (16), as well as most of the individuals from the São Francisco river basin (34), showed an operculum opening extending posteriorly, a deeper nape region, a posteriorly inserted pectoral fin, a posteriorly inserted pelvic fin, and a larger caudal peduncle depth (Fig. 3a).



**Fig. 3** Canonical variable analysis for **a** *Cichlasoma orientale* and **b** *Crenicichla menezesi*. Warped outline graphs demonstrate the phenotypic variation of each canonical variable.

Horizontally, left to right, negative to positive variation. Vertically, bottom to top, negative to positive variation

Among *C. menezesi* populations, CV1 explained 55.60% of the intraspecific shape variation. Almost all the individuals from Paraíba do Norte (20), most of Piranhas-Açu (28) and some individuals from São Francisco (11) and Jaguaribe (10) river basins, exhibited a higher maximum depth, an posteriorly positioned dorsal fin insertion, a shorter snout length and a shorter eye diameter. Indeed, some individuals from the São Francisco (20), Jaguaribe (13), and the majority of Apodi-Mossoró (25) individuals showed a shorter maximum body depth, an anteriorly positioned dorsal fin, an increased snout length, a relatively upturned mouth position, and longer caudal fin.

CV2 explained 24.31% of total shape variation in *C. menezesi*. Most of the individuals from Apodi-Mossoró (15), São Francisco (20), and Paraíba do Norte (19) exhibited smaller heads, with a lower maximum head depth. In addition, an anteriorly positioned dorsal fin and a posteriorly inserted pectoral fin were observed at those specimens. Most of the individuals from Piranhas-Açu (27) and Jaguaribe (17), some from São Francisco (11) exhibited a posteriorly inserted dorsal fin and an anteriorly inserted pectoral fin, as well as a higher head (Fig. 3b).

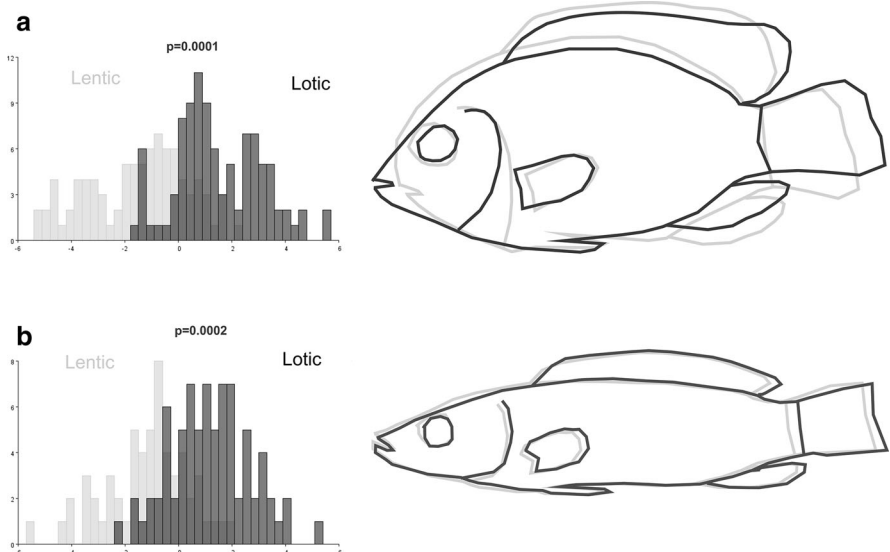
The ANOVA results did not reveal a significant morphological variation between habitats in *C. menezesi*. However, the discriminant analysis, which is more useful to discriminate slight differences in pairwise comparisons than ANOVA, detected a

significant effect of lentic versus lotic habitats on body shape variation for both species. Populations of both *C. orientale* ( $T^2 = 140.15$ ;  $P = 0.0001$ ) and *C. menezesi* ( $T^2 = 78.94$ ;  $P = 0.0002$ ) exhibited similar morphological changes, mainly related to body depth and snout length. *C. orientale* individuals from lentic habitats exhibited a more elliptical body shape, with maximum body depth posteriorly positioned, higher horizontal eye diameter, larger dorsal, anal and caudal fins, anteriorly positioned insertion of pectoral fin and a more sub-terminal mouth position. Individuals of *C. menezesi* from lentic habitats also showed a deeper head, higher horizontal eye diameter, and larger caudal and anal fins. For lotic conditions, a more fusiform-like shape was exhibited for both species, as well as reduced eye diameter, reduced body depth and a longer caudal fin. Additionally, *C. orientale* showed a more terminal mouth position (Fig. 4).

Two different hypotheses were tested using AMOVA. The first explained 79.58% of the observed genetic variation in *C. orientale* being significantly related to genetic structure among basins (Table 2a). However, for *C. menezesi* the major part of the molecular variation was shared among sampling locations within basins (Table 2c). The second hypothesis, testing the molecular variation among habitats, was not significant for both species (Table 2b, d).

The analysis of cytb sequences revealed shared haplotype distribution among populations and habitats

**Fig. 4** Discriminant analysis between lentic and lotic habitats for **a** *Cichlasoma orientale* and **b** *Crenicichla menezesi*. In gray, warped outline graphic of shape variation for lentic habitats. In black, warped outline graphic of shape variation for lotic habitats



**Table 2** “Phi-statistics” and the hierarchical partitioning of genetic variation among/within basins and habitats calculated from AMOVA using  $P$  as distance method

<i>Cichlasoma orientale</i>	% total variation	$\phi$ statistics	$P$
(a)			
Among basins	79.58	$\phi_{CT} = 0.719$	$0.000 \pm 0.000$
Among sampling locations within basins	4.06	$\phi_{SC} = 0.198$	$0.084 \pm 0.008$
Within sampling locations	16.36	$\phi_{ST} = 0.836$	$0.000 \pm 0.000$
(b)			
Among habitats	23.39	$\phi_{CT} = 0.233$	$0.156 \pm 0.011$
Among sampling locations within habitats	60.74	$\phi_{SC} = 0.792$	$0.000 \pm 0.000$
Within habitats	15.87	$\phi_{ST} = 0.841$	$0.000 \pm 0.000$
<i>Crenicichla menezesi</i>	% total variation	$\phi$ statistics	$P$
(c)			
Among basins	27.54	$\phi_{CT} = 0.275$	$0.127 \pm 0.009$
Among sampling locations within basins	42.35	$\phi_{SC} = 0.584$	$0.001 \pm 0.001$
Within sampling locations	30.11	$\phi_{ST} = 0.698$	$0.000 \pm 0.000$
(d)			
Among habitats	18.72	$\phi_{CT} = -0.187$	$0.803 \pm 0.012$
Among sampling locations within habitats	84.03	$\phi_{SC} = 0.707$	$0.000 \pm 0.000$
Within sampling locations	34.69	$\phi_{ST} = 0.653$	$0.000 \pm 0.000$

The partitioning of genetic variation was based on current basins lines.  $P$  values are the probability under the null distribution of having a more extreme  $\phi$  statistics than the observed value by chance

(a) AMOVA results for *C. orientale* among basins; (b) AMOVA results for *C. orientale* among habitats; (c) AMOVA results for *C. menezesi* among basins; (d) AMOVA results for *C. menezesi* among habitats

for both species (Fig. 5). In *C. orientale*, the analysis detected five haplotypes, with two appearing with particularly high frequency. One of these haplotypes comprised specimens from Paraíba do Norte, Piranhas-Açu, and São Francisco basins (Haplotype 1). The other major haplotype comprised specimens from Jaguaribe, Apodi-Mossoró, and one individual of Paraíba do Norte basins (Haplotype 4). The other three haplotypes were unique to specimens from Apodi-Mossoró basin (Haplotype 3 and 5), and Piranhas-Açu basin (Haplotype 2) (Fig. 5a). In *C. menezesi*, three haplotypes were detected. The most frequent one (Haplotype 1) was present in all five isolated basins (Apodi-Mossoró, Jaguaribe, Paraíba do Norte, Piranhas-Açu, and São Francisco). The other haplotypes were unique to Jaguaribe basin (Haplotype 2 and 3) (Fig. 4c). Besides most of the sequences included were from lotic habits for both species, lentic habitats individuals shared the major haplotypes with lotic habitats individuals. In *C. orientale*, the Haplotype 1 comprised 13 individuals of lentic habitats, and

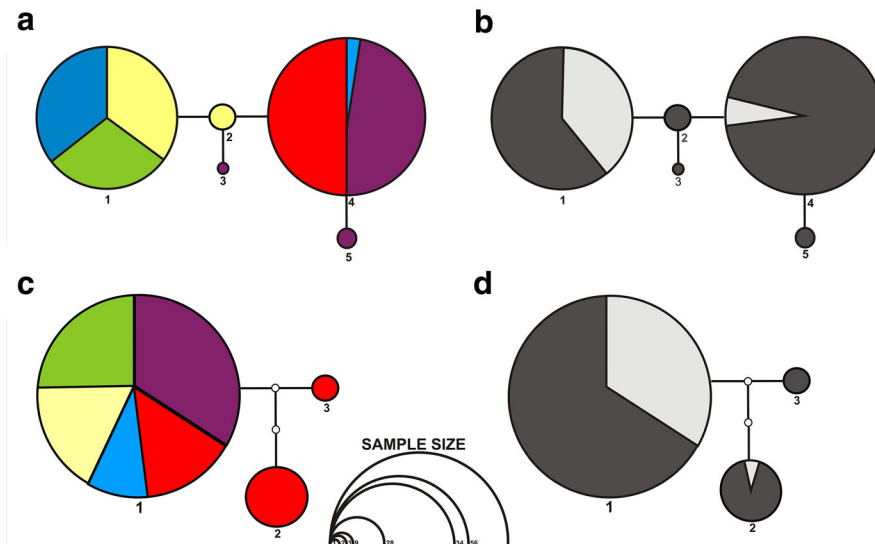
the Haplotype 4, three of them (Fig. 5b). For *C. menezesi*, the Haplotype 1 comprised 17 individuals from lentic habitats while the Haplotype 2 comprised one (Fig. 5d) (Table S2).

The Mantel test showed no significant relationship between morphological and genetic data for either species ( $R^2 = 0.077$ ;  $P = 0.503$  for *C. orientale*;  $R^2 = 0.184$ ;  $P = 0.801$  for *C. menezesi*), suggesting that the current variation is not related to ancient lineage isolation among northeastern Brazil basins (Table 3).

## Discussion

Allopatric diversification among isolated basins is the usual mechanism of divergence in Neotropical freshwater fishes (Albert & Reis, 2011). Our results revealed significant morphological variation among populations of *C. orientale* and *C. menezesi* from isolated basins involved in the São Francisco





**Fig. 5** Haplotype networks of cytb gene for **a** *Cichlasoma orientale* among basins **b** *Cichlasoma orientale* among habitats **c** *Crenicichla menezesi* among basins **d** *Crenicichla menezesi* among habitats. Numbers represent the haplotype number. Purple Apodi-Mossoró river basin individuals; Red Jaguaribe

river basin individuals; Blue Paraíba do Norte river basin individuals; Yellow Piranhas-Açu river basin individuals; Green São Francisco river basin individuals; Light gray Lentic habitats; Dark gray Lotic habitats

interbasin water transfer project (Tables 1 and 3; Fig. 3). Some studies with Neotropical cichlids have demonstrated allopatric diversification among drainages (Ready et al., 2006; Musilová et al., 2008; Ríčan & Kullander, 2008). Besides both genetic and morphological comparisons among basins were significant for *C. orientale* (only for morphological data of *C. menezesi*), our data showed no significant correlation between morphological and genetic variation for both species. It is likely that significant genetic and morphological distances among basins for *C. orientale* were not related due to the low genetic variation found using this marker. However, since the objective was to test the genetic structure among basins (and check whether it is correlated to morphological variation among basins), the marker used seems to answer this question appropriately. Furthermore, morphological traits fixation and genetic structure can have different patterns, since scenarios of rapid morphological responses (mainly through phenotypic plasticity) could mask the genotypic signal of genetic isolation among lineages, resulting in different signals between morphological and molecular data (Hull et al., 2010). Our data also corroborate similar responses among

**Table 3** Matrix used in Mantel test to correlate molecular data (*P* distance) and morphological data (Malahanobis distance) among basins (with habitats combined within basins)

	APO	JAG	PAR	PAC	SFR
(a)					
APO	–	2.106***	2.465***	2.003***	2.206***
JAG	0	–	1.919***	1.691**	1.966*
PAR	0.002	0.002	–	2.431***	2.262**
PAC	0.002	0.002	0	–	1.899**
SFR	0.002	0.002	0	0	–
(b)					
APO	–	2.35***	3.463***	2.881***	2.085***
JAG	0.002	–	2.439***	1.654*	1.4587 <sup>ns</sup>
PAR	0	0.002	–	2.328***	2.264***
PAC	0	0.002	0	–	1.779**
SFR	0	0.002	0	0	–

Below diagonal, *P* distance values; above diagonal, Malahanobis distance values

APO Apodi-Mossoró basin; JAG Jaguaribe basin; PAR Paraíba do Norte basin; PAC Piranhas-Açu basin; SFR São Francisco basin; ns not significant

\* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* < 0.001

(a) Matrix data for *C. orientale*; (b) Matrix data for *C. menezesi*

species for each habitat, suggesting that the current shape variation of *C. orientale* and *C. menezesi* populations in northeastern Brazil could be environmentally driven.

The Mid-Northeastern Caatinga ecoregion has an aquatic regime characterized by constant drying and flooding (Rocha et al., 2012). Our discriminant analysis results also revealed a significant shared morphological variation between individuals collected in lentic and lotic habitats for both species (Fig. 4). Given that no significant genetic structure was found among habitats (Table 2b, d), and our haplotype network revealed shared distribution of habitats among different basins (Fig. 5b, d), the data suggest that, for both species, there is additional environmental impact upon morphological diversification within river basins that are related to different water flow regimes.

Many studies report the independent evolution of similar phenotypes in separate but similar environments. This convergence of phenotypes implies, with some caveats, a strong deterministic environmental role (Hendry, 2013). Changes in the positions of the maximum body depth usually reflect variations in the swimming capacity/strategy (Langerhans et al., 2003). A deeper body and larger dorsal, anal and caudal fins generally indicate a greater potential in maneuverability and stabilization in deflections (Sampaio et al., 2013). This is consistent with the burst swimming pattern associated with lentic environments (Langerhans & Reznick, 2010; Crispo & Chapman, 2011), and fits with our results. Other studies have found similar shape patterns for fishes in lentic habitats (Langerhans et al., 2007; Langerhans, 2008; Haas et al., 2010; Franssen et al., 2012), including Neotropical cichlids (Langerhans et al., 2003; Sampaio et al., 2013). While not necessarily directly related to swimming capacity, other morphological differences found are probably related to lentic habitats (Figs. 3 and 4). Deeper heads may represent a morphological response related to the larger size of gill arches in waters with low levels of dissolved oxygen (Langerhans et al., 2007; Crispo & Chapman, 2011). Both species also exhibited an increased eye diameter in lentic habitats, which is likely related to the low visibility in lentic habitats that results from floating macrophytes and intense sedimentation (Sampaio et al., 2013).

A more streamlined body shape, found in both species here for lotic conditions, reduces water

resistance and, consequently, the energetic expenditure to maintain position in flowing water (Langerhans, 2008). This shape commonly reflects a sustained swimming strategy, typically employed by individuals swimming in high flow water environments (Langerhans 2008, Langerhans & Reznick, 2010). This kind of shape variation in fishes for lotic freshwater environments is a predictable and well-studied pattern (for a review, Langerhans, 2008).

Changes in snout length and mouth position among habitats could reflect variations in feeding behavior, diet type, and foraging mode (Langerhans et al., 2003). *C. orientale* is a predominantly omnivorous mid-water cichlid with a plastic foraging behavior that changes with resource availability (Gurgel et al., 2002, 2005). It may be that *C. orientale* individuals from lentic habitats have responded to changes in macroinvertebrate availability that have occurred in this region (Rocha et al., 2012), and developed a feeding niche that is specialized to bottom detritus or invertebrates in lentic habits (Fig. 4a). However, more studies about the diet of this species are necessary to elucidate the relationship between resource availability and variation in feeding structures. *Crenicichla menezesi* is a predominantly carnivorous generalist species (Gurgel et al., 2002, 2005). Our results showed a slight difference in snout length in lentic habitats, a finding which may relate to attack speed or accuracy, as has previously reported in cichlids (Higham et al., 2007; Sampaio et al., 2013). This species also exhibited a longer caudal fin and a higher caudal peduncle, which are functionally related to higher maneuverability, movement stabilization, and burst displacements (Oliveira et al., 2010) (Fig. 4b). Sampaio et al., (2013), in an ecomorphological view of *Crenicichla britskii* Kullander 1983, found similar results in swimming and prey capture strategy between environments, reinforcing the idea of morphological variation being predictive of ecological behavior in *Crenicichla* species.

Although *C. orientale* and *C. menezesi* belong to the same subfamily, these genera are relatively distant from each other in phylogenetic terms (López-Fernández et al., 2010). However, the shape responses of both species to ecological conditions related to water flow regimes (which has a high influence in many other aquatic variables) were similar. The intraspecific shared shape changes of these species could be environmentally driven, potentially through

phenotypic plasticity action. Three major factors support a role for plasticity: (1) There was no significant genetic structure differences between habitats for either species (Table 2b, d; Fig. 5b, d); (2) For both species, multiple sampling locations in different isolated rivers, which differ in their ecological histories, showed similar morphological responses to water flow regime; (3) With respect to fixation of phenotypic traits, it is unlikely that stochastic processes have acted in the same way for different populations. However, it is not possible to discard multiple scenarios of local adaptation shaping the morphological variation for both species, and a multilocus DNA study (i.e. RAD-seq), including locus under selection, should be employed to understand the evolutionary pathways behind the lotic and lentic shapes.

Phenotypic plasticity allows for rapid shifts between peaks and valleys in an adaptive landscape without compromising individual fitness. Such plasticity can also contribute to population divergence (Pfennig et al., 2010), especially when multiple adaptive peaks are possible, as is especially true in variable environments (Price et al. 2003). Both species have already demonstrated the use of reproductive strategies characterized by a set of physiological adaptations for semi-arid conditions and intermittent water flow regime (Gurgel et al., 2011; Araújo et al., 2012). The morphological variations may be the responses to the seasonal modifications in water flow regimes (lotic to lentic, and vice versa), which is a remarkable aquatic behavior that is characteristic of Northeastern Brazil (Rocha et al., 2012).

Determining the limits of phenotypic plasticity and the evolutionary potential of traits that affect survival and fitness will be critical to assessing biological responses to anthropogenic activities (Reed et al., 2011). As argued by Malthick & Medeiros (2006), one of the most important issues in the conservation of freshwaters in semi-arid Brazil is the recognition that the diversity of this region is closely associated to natural patterns of water flow and hydrological disturbances. Our results indicate significant inter-basin morphological variation, as well as a prominent influence of water flow regime upon morphological variation in Mid-Northeastern Caatinga. This last environmental condition has been reported as an important component of distribution and richness of macroinvertebrates in this region (Rocha et al., 2012), as well as fish diversity and stability (Medeiros &

Maltchik, 2001; Medeiros et al., 2006). Changes in water flow regime are usually linked with interbasin water transfer projects (Meador, 1992; Collen et al., 2013). Thus, the São Francisco interbasin water transfer project may affect the adaptive potential of phenotypic plasticity in response to water flow regime, mainly in areas that are directly receiving waters from the two axes, as well as the epigenetic mechanisms that are source of its variation. Further, plasticity has limits that are regulated by the relationship between costs and fitness of individual plasticity as modulated by the frequency and nature of environmental changes (Reed et al., 2011). If there are high energetic costs associated with plastic responses, selection may favor non-plastic organisms, especially in relatively stable environments where changes are infrequent and/or not predictable anymore (Svanbäck et al., 2009).

According to Albert & Reis (2011), 54.6% of Neotropical freshwater fishes are restricted to a single ecoregion, underlining the extent to which historical and ecological conditions constrain lineage distributions according to major landscapes. Although the National Integration Ministry had reported that the environmental impacts of the São Francisco interbasin water transfer will be minimal (Pittock et al., 2009), this work will represent a large inter-ecoregion exchange between aquatic fauna and gene flow among lineages that are naturally isolated and evolving independently. Our haplotype network revealed a shared lineage history among basins, which may represent a scenario of incipient differentiation as lineage sorting or ancestral polymorphism of populations, but some unique basin lineages (mainly Apodi-Mossoró, Jaguaribe and Piranhas-Açu) were found (Fig. 4). In Brazil, cases of genetic homogenization in fishes (Bellafrente et al., 2010; Blanco et al., 2009; Lima & Caires, 2011; Peres et al., 2012) have already been linked to interbasin water transfer projects. Possible gene flow between isolated populations potentially represents unpredictable and irrecoverable losses of intraspecific diversity, evolutionary potential and information about the lineage history in Mid-Northeastern Brazil.

The present study represents the first evolutionary examination of the aquatic species involved in the São Francisco interbasin water transfer project, and reports significant morphological variation among both *C. orientale* and *C. menezesi* populations, potentially related to the natural drying and flooding regime from

Northeastern Brazil. This study provides a base line approach of morphological and molecular variation before the artificial connection between basins is finished. An additional phylogeographic study including more individuals along the distribution of both species is currently being done to elucidate how geological and climatic events that regulate basin dynamics in northeastern Brazil have influenced lineage distribution in these species. The current findings highlight the increasing necessity of studies on the evolutionary biology using integrative approaches that are able to evaluate historical and ecological scenarios of current phenotypic variation, as well as the importance of such factors for maintenance of the evolutionary potential of species.

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#### Compliance with ethical standards

**Conflict of Interest** The authors declared there is no conflict of interest about the data on this manuscript.

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