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Genetic and morphological analysis of *Herichthys pantostictus* (Cichliformes: Cichlidae) populations that showed mitochondrial DNA introgression

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One factor that could explain the great diversity of cichlids is genetic introgression. In Mexico, there are 23 genera of cichlids; one of the most diverse is the genus *Herichthys*, which comprises 11 valid species. *Herichthys pantostictus* and *H. carpintis* are two species with a broad distribution in the Pánuco-Tamesí basin, where they often occur in sympatry. This study uses mitochondrial and nuclear sequences and morphological features to assess possible hybridization between these species. The phylogeny obtained with mitochondrial markers recovered the haplotypes of both species as paraphyletic, with a group of *H. pantostictus* haplotypes nested within the *H. carpintis* haplotypes. In contrast, the phylogeny obtained with the nuclear marker *ITS-1* recovered the haplotypes of both species as reciprocally monophyletic. However, the traditional morphological features differed significantly between populations, but with a high degree of overlap, as did the geometric morphometrics, where there were no differences. In conclusion, we could detect signals of an ancient introgression, but did not observe morphological differences in the hybrid populations.

Keywords: Cichlids, Geometric morphometrics, Hybrids, Phylogeny, Sympatry.

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Uno de los factores que permite explicar la gran diversidad de cíclidos es la introgresión genética. En México, hay 23 géneros de cíclidos; uno de los más diversos es el género *Herichthys*, que tiene 11 especies válidas. *Herichthys pantostictus* y *H. carpintis* son dos especies con amplia distribución en la cuenca Pánuco-Tamesí, donde frecuentemente se encuentran en simpatría. En este estudio, utilizamos secuencias mitocondriales y nucleares, así como datos morfológicos, para caracterizar la presencia de híbridos entre estas especies. La filogenia obtenida con marcadores mitocondriales recuperó los haplotipos de ambas especies como parafiléticos debido a la inclusión de un grupo de haplotipos de *H. pantostictus* anidados dentro de los haplotipos de *H. carpintis*. Por el contrario, la filogenia con el marcador nuclear *ITS-1* recuperó los haplotipos de ambas especies como recíprocamente monofiléticos. Por otro lado, los caracteres morfológicos tradicionales mostraron diferencias significativas entre las poblaciones, pero con un alto grado de solapamiento, al igual que en la morfometría geométrica. En conclusión, encontramos señales de una introgresión genética antigua, pero sin diferencias morfológicas perceptibles en las poblaciones híbridas.

Palabras clave: Cíclidos, Filogenia, Híbridos, Morfometría geométrica, Simpatría.

INTRODUCTION

Hybridization is more common in fish than in other vertebrate groups (Hubbs, 1955; Salzburger *et al*., 2002). Genetic introgression is considered a potential generator of diversification. However, allopolyploidy represents one of the main obstacles to the formation and establishment of hybrid populations, since it makes the offspring infertile or with lower fitness than their parents (Stebbins, 1959; Soltis, Soltis, 1995; Chenuil *et al*., 1999). In animals, most cases of hybridization are not necessarily associated with a chromosomal mismatch, since the process usually occurs between closely related species; therefore, in most cases, hybrids have the same number of chromosomes as their parental species (DeMarais *et al*., 1992; Dowling, Secor, 1997). Hybridization has been attributed to factors such as external fertilization mechanisms, lack of ethological isolation, differences in the abundance of parental species, decreased habitat complexity, and susceptibility to secondary contact between species of recent origin (Hubbs, 1955; Seehausen, 2004). These processes can give rise to temporary or permanent changes in the genetic pool of the populations involved, with important evolutionary consequences (Harrison, 1986; Dowling, Secor, 1997; Seehausen, 2004; Mallet, 2005; Taylor *et al*., 2006; Parnell *et al*., 2012).

It has been hypothesized that introgression could transfer complete blocks of genes that stabilize each other and, along with local selective pressures, lead to the development of new niches and rapid speciation (Anderson, 1948; Anderson, Stebbins, 1954; Geiger *et al*., 2010; Elmer, Meyer, 2011). Cichlids represent one group of freshwater fish with the greatest richness and diversity in tropical regions of the world (Turner, 2007), which has been attributed to factors such as habitat segregation, trophic specialization, sexual selection, and hybridization (Burress, 2015). In particular, the neotropical cichlids of Middle America comprise about 40 genera, of which 23 are distributed in Mexico (Rican *et al*., 2016). One of them is the genus *Herichthys* Baird & Girard, 1854, the only representative of the family that is distributed to the northeast of Punta del Morro (Veracruz), which has recently been the subject of numerous studies (Pérez-Miranda *et al*., 2018, 2019, 2020). The genus *Herichthys* comprises 11 valid species, most of which have a restricted distribution, except *H. pantostictus* (Taylor & Miller, 1983) and *H. carpintis* (Jordan & Snyder, 1899), which show a broad distribution in the Pánuco-Tamesí basin, where they are frequently found to occur sympatrically (Pérez-Miranda *et al*., 2018).

Both species have a high degree of overlap in morphological features, including the shape of the body and head (Mejía *et al*., 2015; Pérez-Miranda *et al*., 2018); thus, the main differences that allow their discrimination are colorations. *Herichthys pantostictus* usually has a base coloration that varies from gray to yellow with small brown dots on the body and head. In contrast, *H. carpintis* usually has a base coloration that varies from gray to green with large and iridescent blue dots all over the body (Fig. 1). Their nuptial colorations are also entirely different. In *H. pantostictus*, the lower middle of the body is a darkened area that extends from the lower edge of the eye leaving a small uncolored space between the pelvic fins and the anal fin. In contrast, in *H. carpintis*, three-quarters of the body is darkened, leaving only the upper part of the head without color (Pérez-Miranda *et al*., 2018).

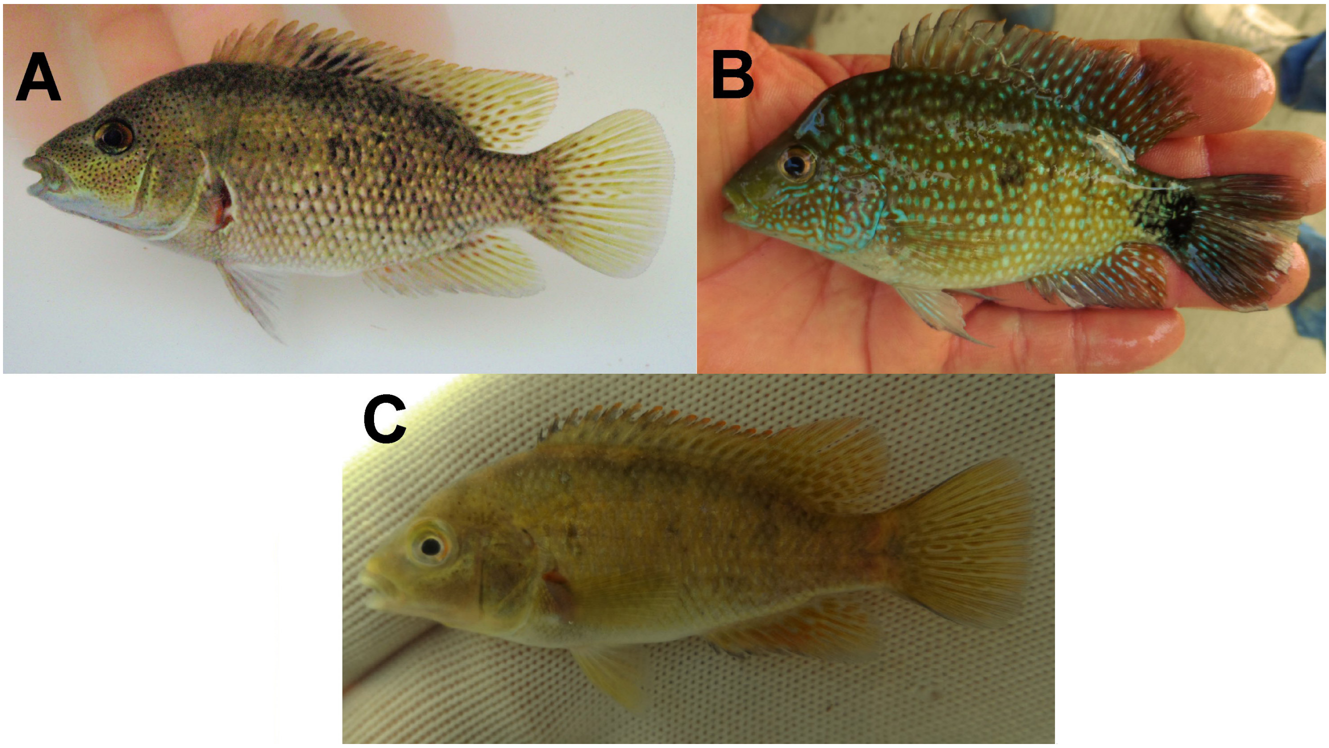

FIGURE 1 | Typical morphotype of *Herichthys pantostictus* (**A**), typical morphotype of *H. carpintis* (**B**), and putative hybrid (**C**).

Preliminary DNA barcoding studies suggest that some haplotypes of specimens identified as *H. pantostictus* (hereinafter referred to as putative hybrids) are recovered as a monophyletic group nested within the haplotypes of *H. carpintis* (León-Romero *et al*., 2012; Mejía *et al*., 2015). Subsequent complementary studies using the sequence of the mitochondrial cytochrome b (*cytb*) marker support the findings previously established with the mitochondrial cytochrome c oxidase subunit I (*COI*) marker (Pérez-Miranda *et al*., 2018). Different hypotheses could explain our previous findings. For example, it has been reported that incomplete lineage sorting may explain the genetic discordance observed in some groups of cichlids in Africa; a process that, coupled with hybridization events, could explain their great diversity (Schwarzer *et al*., 2015; Astudillo-Clavijo *et al*., 2023). An alternative scenario is a hybridization zone between both parental species, as has been reported in other species of the genus *Herichthys* in Northeastern Mexico (Hulsey *et al*., 2016). This study comparatively analyzes *H. pantostictus* and *H. carpintis* using mitochondrial and nuclear markers and morphological features to evaluate possible hybridization between them.

MATERIAL AND METHODS

Phylogenetic analysis. First, 328 sequences of the mitochondrial *COI* and *D-Loop* markers generated in previous studies were used (León-Romero *et al*., 2012; Mejía *et al*., 2015; Pérez-Miranda *et al*., 2018; Pérez-Miranda *et al*., 2023). Then, once the origin of the *H. pantostictus* haplotypes nested within the *H. carpintis* haplotypes had been verified, the internal transcribed spacer 1 (*ITS-1*) was amplified from selected populations to cover the geographic distribution of the two parental species and the putative hybrids. In total, 25 *H. pantostictus* individuals, 26 *H. carpintis* individuals, and 18 potentially hybrid individuals were amplified (Fig. 2; Tab. **S1**). Note that the putative hybrids were identified as *H. pantostictus* using taxonomic keys for the members of the genus; therefore, the specimens considered as putative hybrids in this study belong to those populations of *H. pantostictus* whose haplotypes are nested within *H. carpintis* (Fig. 2).

To examine the *ITS-1* marker, DNA was extracted according to the protocol of Aljanabi, Martínez (1997). Next, the *ITS-1* locus was amplified via polymerase chain reactions (PCRs) using primers designed for this study: CAB-F (5'-TTTCCGTAGGTGAACCTG-3') and CAB-R (5'-CCGCTAAGAGTCGTATTGT-3'). The reaction mix contained 1× PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer, 40 ng of template DNA, and 1 U of GoTaq (Invitrogen). The amplification conditions were as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were purified, and both strands were sequenced using the Sanger method. The sequences were edited in Seaview (Gouy *et al*., 2010) and aligned in Clustal X 2.0 (Larkin *et al*., 2007). The nuclear *ITS-1* sequences generated in this study are deposited in GenBank under accession numbers PP987868 to PP987903.

For the case of the mitochondrial markers, three sequences from other species of the genus (*Herichthys pame* (De la Maza-Benignos & Lozano-Vilano, 2013), *H. steindachneri* (Jordan & Snyder, 1899), and *H. tepehua* (De la Maza-Benignos, Ornelas-García, Lozano-Vilano, García-Ramírez & Doadrio, 2015) were added as external groups. For

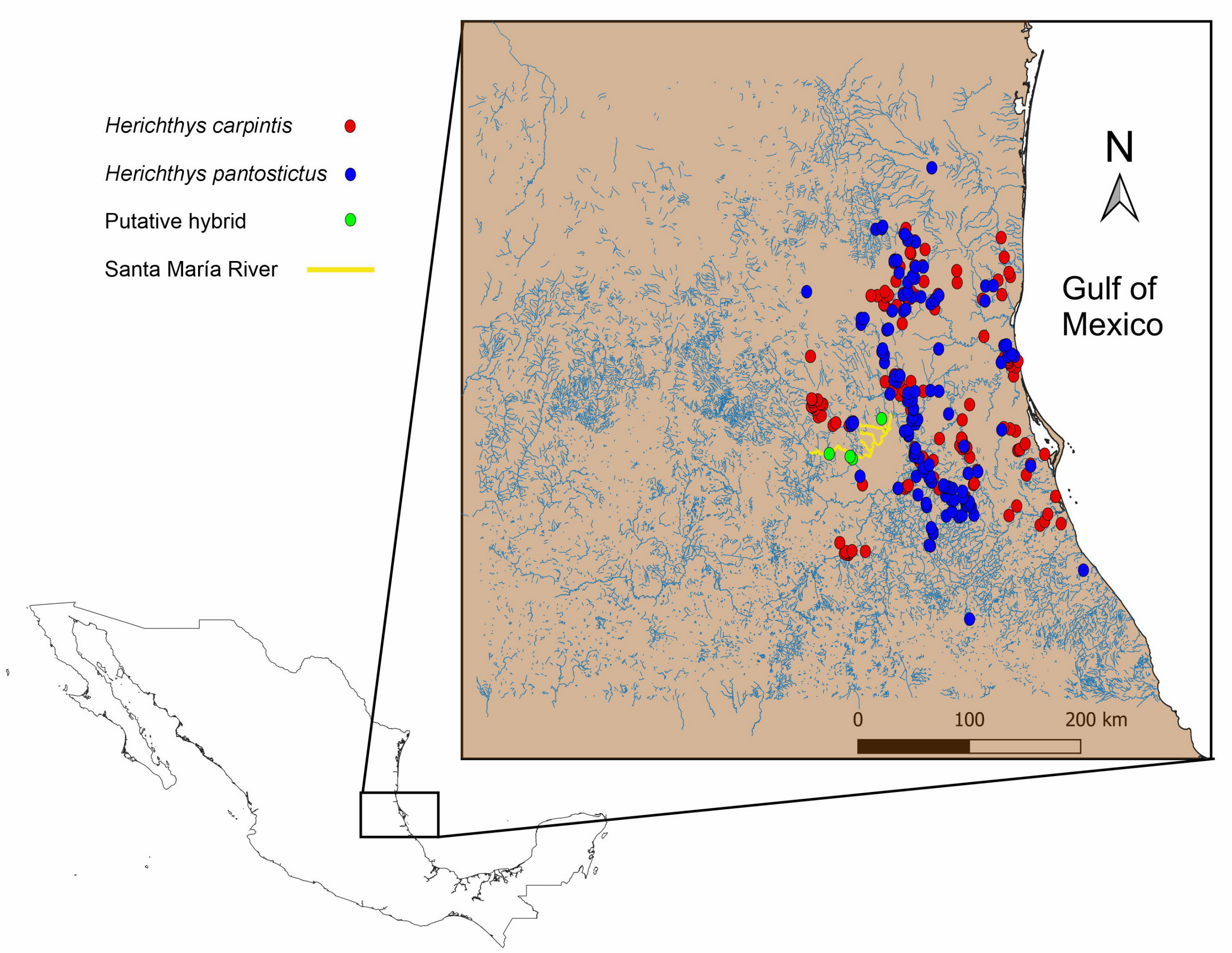

FIGURE 2 | Geographic distribution map of *Herichthys carpintis* (red), *H. pantostictus* (blue), and putative hybrids populations (green).

the nuclear *ITS-1* marker, it was not possible to include sequences from other species because none are available. The aligned sequences were used in a phylogenetic analysis using the BEAST program v. 1.7.5 (Drummond, Rambaut, 2007) with the following parameters: chain length of 10 million generations, sampling every 10,000 generations, a GTR + I + G substitution model chosen according to the Akaike information criterion of jModelTest v. 2.1.10 (Posada, 2008), and a strict molecular clock with a secondary calibration based on the previously reported divergences times between the parental species (Pérez-Miranda *et al*., 2020). This analysis aimed to estimate the divergence times between the different populations of the parental species and the putative hybrids. Four independent runs were conducted, which were analyzed using Tracer v. 1.7.2 (Rambaut *et al*., 2018) to verify the convergence of the analyses. Then, the resulting trees were combined using LogCombiner v. 1.7.5 (Suchard *et al*., 2018), and a consensus tree at 50% with a burn-in of 25% was constructed in TreeAnnotator v. 1.7.5 (Suchard *et al*., 2018). Finally, the consensus tree was edited using FigTree v. 1.4.4 (Suchard *et al*., 2018) to estimate divergence times and highest posterior density (HPD).

BAPS and gene flow. Assuming that all individuals belong to a single taxonomic entity, we assessed the number of genetic clusters (*k*; hereinafter referred to as populations) using the "optimise.baps" option in the *fastbaps* library (Tonkin-Hill *et al*., 2019) of the R statistical software v. 4.0.4 (RStudio Team, 2020). After the genetic clusters were defined, we evaluated the levels of gene flow among them using the Bayesian approach implemented in migrate-n v. 4.4.2 (Beerli, 1998; Beerli, Felsenstein, 2001). We used a static heating scheme with four temperature chains (1, 1.5, 3, and 1,000,000), each analysis comprised 10,000,000 genealogies that were sampled every 1000 generations after a 10% burn-in. We used the full matrix model as a null hypothesis and included three different gene flow models for each mitochondrial marker; putative hybrids received migrants from both species, and putative hybrids received migrants either from *H. pantostictus* or *H. carpintis* exclusively. Finally, we compared the gene flow models using a Bayes factor test with the Bezier approach's marginal likelihood (Beerli *et al*., 2019) using the "BF" function in R's *mtraceR* library (Pacioni *et al*., 2015).

Traditional morphometrics. The traditional morphological analysis included 691 specimens deposited in the Colección Nacional de Peces Dulceacuícolas Mexicanos de la Escuela Nacional de Ciencias Biológicas (ENCB-P), considering the complete distribution of the parental species: 383 correspond to the species *H. pantostictus*, 239 to *H. carpintis* (Pérez-Miranda *et al*., 2018), and 69 to the putative hybrids according to the results of the molecular analysis. In the ENCB-P, several specimens were collected in the same localities as the suspected hybrids, which were included in the morphological analyses (Tab. **S1**). The locations of the specimens detected as potential hybrids correspond to a polygon that includes populations in the Santa María River, which drains the states of Querétaro, San Luis Potosí, and Guanajuato (Fig. 2).

To record the measurements, we selected specimens whose standard length (SL) was >40 mm to ensure that they were adults. No distinction was made between females and males because nuptial coloration was absent in most specimens. In total, 27 morphometric features were recorded with a digital caliper with a precision of 0.01 mm and 12 meristic features, following the work of Pérez-Miranda *et al.* (2018).

The morphometric variables recorded were as follows: total length (TL), standard length (SL), total length of the anal fin (LAF), total length of the dorsal fin (LDF), length of the dorsal fin of spines (DFE), length of the dorsal fin of rays (DFR), length of the anal fin of spines (AFE), length of the anal fin of rays (AFR), length of the pectoral fins (LPF), length of the pelvic fins (LVF), predorsal length (PDL), preanal length (PAL), postorbital length (POL), length of the upper maxilla (UML), length of the lower maxilla (LLM), length of the caudal peduncle (LCP), length of the dorsal fin at its base (LDB), length of the anal fin at its base (LAB), head length (HLE), snout length (SNL), length of the ascending premaxillary process (LPP), length of the post ascending premaxillary process (PPP), distance between anal fin and the base of the pelvic fins (DBF), body height (BHE), height of the caudal peduncle (HCP), eye diameter (EYD) and interocular distance (IOD) (Fig. 3).

The meristic variables recorded were as follows: the number of spines in the dorsal fin (DS), the number of rays in the dorsal fin (DR), the number of spines in the anal fin (AS), the number of rays in the anal fin (AR), the number of rays in the pectoral fin (PR), the number of rays in the pelvic fin (PFR), the number of gill rakers in the

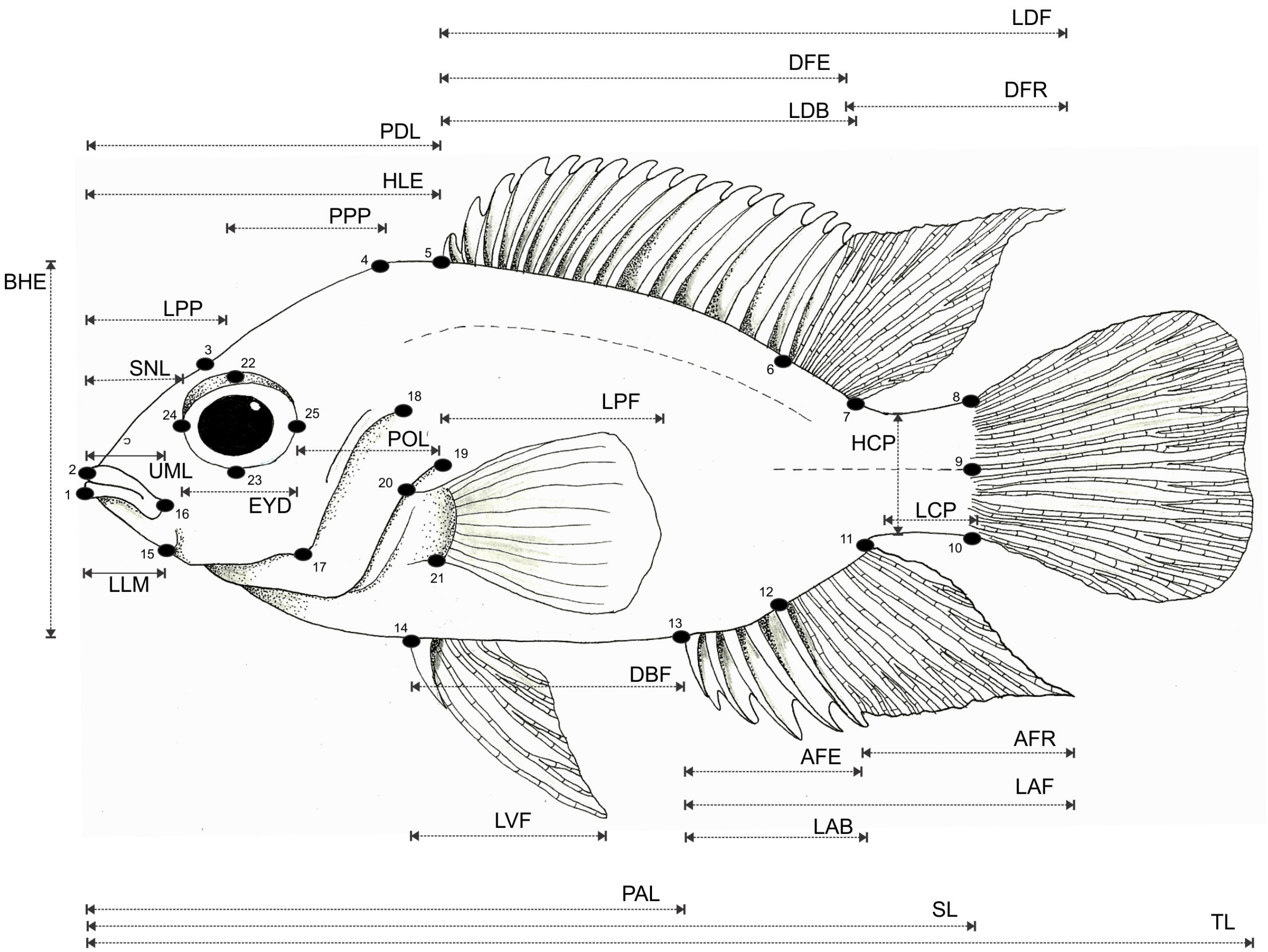

FIGURE 3 | Morphometrical measures considered in this study. Total length (TL), standard length (SL), total length of the anal fin (LAF), total length of the dorsal fin (LDF), length of the dorsal fin of spines (DFE), length of the dorsal fin of rays (DFR), length of the anal fin of spines (AFE), length of the anal fin of rays (AFR), length of the pectoral fins (LPF), length of the pelvic fins (LVF), predorsal length (PDL), preanal length (PAL), postorbital length (POL), length of the upper maxilla (UML), length of the lower maxilla (LLM), length of the caudal peduncle (LCP), length of the dorsal fin at its base (LDB), length of the anal fin at its base (LAB), head length (HLE), snout length (SNL), length of the ascending premaxillary process (LPP), length of the post ascending premaxillary process (PPP), distance between anal fin and the base of the pelvic fins (DBF), body height (BHE), height of the caudal peduncle (HCP), eye diameter (EYD) and interocular distance (IOD).

first arch (GR), the number of scales in a longitudinal series (SLS), the number of circumpeduncular scales (CS), the number of scales in the first portion of the lateral line (SFLL), the number of scales in the second portion of the lateral line (SSLL), and the total number of scales in the lateral line (TSLL).

The morphometric variables were transformed using two approaches to reduce the bias associated with fish size. First, the variables were standardized as proportions of the SL and then subjected to an analysis of variance (ANOVA). Second, the data were standardized using Mosimann's method (Butler, Losos, 2002) and then subjected to an analysis of covariance (ANCOVA). The meristic variables were analyzed using a Kruskal-Wallis test. Tukey's multiple comparison test was used to determine significant differences between the three groups in all analyses. All these analyses were conducted using the Statistica software v. 10 (Statsoft Inc.). Additionally, the variations in the meristic and morphometric variables between groups (parental and putative hybrids) were explored using a principal component analysis (PCA) of each data set using PAST v. 4.15 (Hammer, Harper, 2001).

Geometric morphometrics. This analysis included 728 specimens obtained from the ENCB-P: 315 belong to *H. pantostictus*, 364 to *H. carpintis*, and 49 to putative hybrids (Tab. **S1**). Each specimen was photographed from the left side with a digital camera. Next, 25 anatomical points were recorded to describe the shape of the body, according to Mejía *et al.* (2015) (Fig. 3). Then, the photographs were analyzed in the tpsDig program v. 2.05 (Rohlf, 2006) to generate the Bookstein coordinates. Next, to correct the curvature effect caused by the deformation of the specimens due to the preservation method, a regression analysis was conducted with tpsUtil v. 1.26 (Rohlf, 2004) using anatomical points 2, 9, 18, and 20 as the reference. Then, the coordinates were subjected to a generalized Procrustes analysis with the "gpagen" function in R's "geomorph" package (Baken *et al.*, 2021). Finally, the distances obtained from the generalized Procrustes analysis were used as descriptors of the shape of the body through a PCA using the "gm.prcomp" function in R's "geomorph" package (Baken *et al.*, 2021) and a canonical variate analysis to maximize the differences between the groups and capture the dispersion of individuals in the multivariate space using MorphoJ v. 1.08.02 (Klingenberg, 2011).

RESULTS

Phylogeny. The phylogenetic analysis of both mitochondrial markers yielded similar results. The estimated age of divergence between *H. carpintis* and *H. pantostictus* was 13.4 Ma for the *COI* marker and 13.5 Ma for the *D-Loop* marker. The *H. carpintis* haplotypes were recovered as a paraphyletic group (Fig. 4), while the *H. pantostictus* haplotypes were recovered as a polyphyletic group. The haplotypes of the putative hybrids were recovered as a monophyletic group nested within the *H. carpintis* haplotypes, with an estimated age of divergence of 1.2 Ma for the *COI* marker and 1.3 Ma for the *D-Loop* marker (Fig. 4). In contrast, the phylogenetic tree constructed from the nuclear *ITS-1* marker provided an estimated age of divergence between species of 5.9 Ma. The haplotypes of each parental species were recovered as reciprocally monophyletic, and the haplotypes of the putative hybrids were nested within the rest of the *H. pantostictus* haplotypes (Fig. 4). Therefore, these results suggest an ancient asymmetric hybridization process between *H. carpintis* and *H. pantostictus*.

BAPS and gene flow. The Bayesian cluster analysis recovered four genetic clusters for the mitochondrial *COI* marker. The first comprised 22 haplotypes of *H. carpintis* and all haplotypes of the putative hybrids, the second comprised 57 haplotypes of *H. carpintis*, the third comprised 85 haplotypes of *H. carpintis*, and the fourth comprised 38 haplotypes of *H. pantostictus* (Fig. 5). Five genetic clusters were recovered for the mitochondrial *D-Loop* marker. The first comprised 54 haplotypes of *H. carpintis* and all

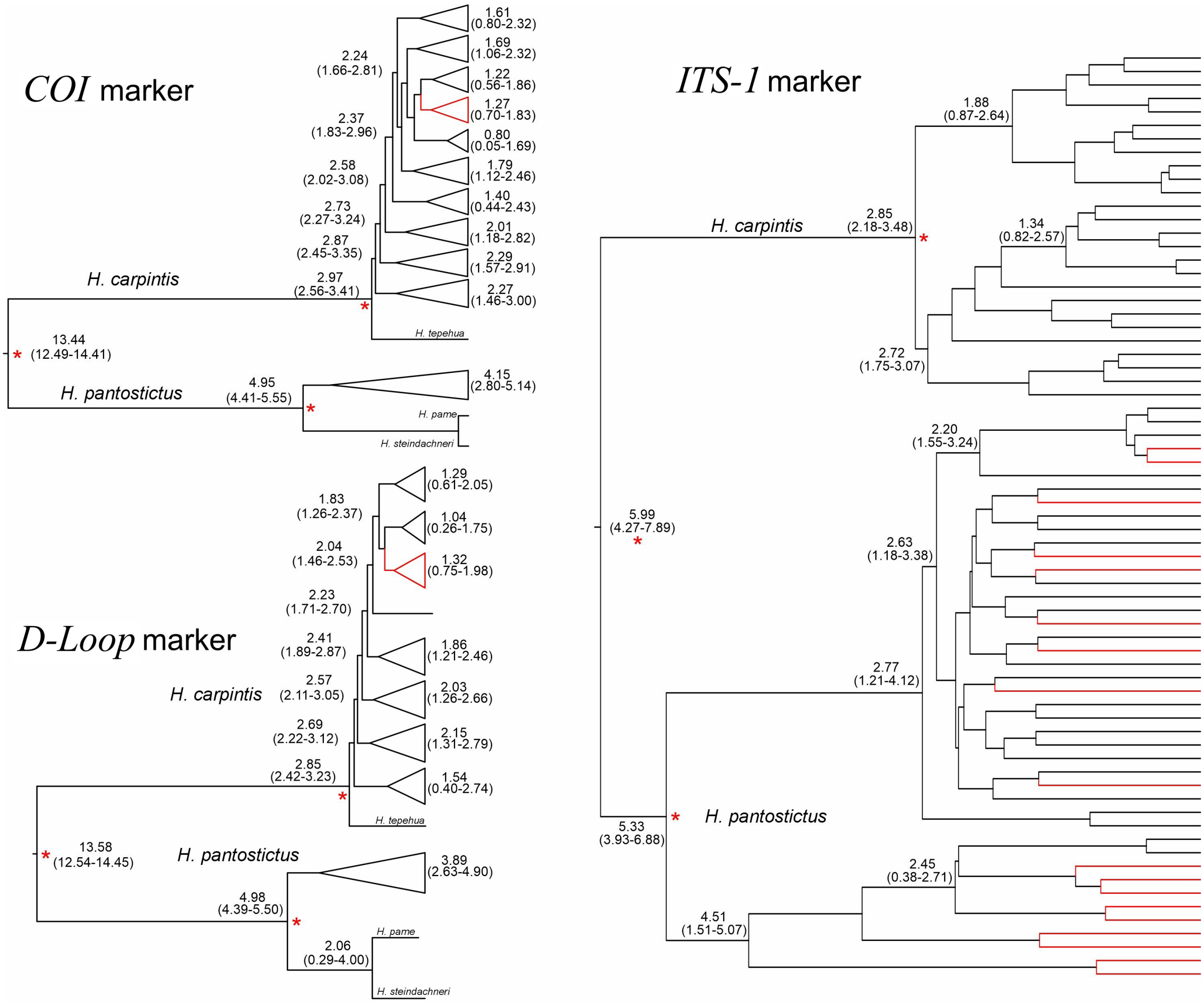

FIGURE 4 | Phylogeny and molecular clock evidencing the conflict between mitochondrial (*COI* and *D-Loop*) and nuclear (*ITS-1*) molecular markers. At each node, the estimated age in millions of years is accompanied by the HPD in parentheses. The red asterisks represent nodes with posterior probability > 0.95.

haplotypes of the putative hybrids; the second, third, and fourth comprised 14, 8, and 2 haplotypes of *H. carpintis*, respectively (the third and fourth were excluded from the Migrate analysis due to their small sizes); finally, the fifth comprised 49 haplotypes of *H. pantostictus* (Fig. 5). The analysis of marginal likelihood using Bayes factor supports that the gene flow was unidirectional from *H. carpintis* clades to the putative hybrid populations (Tab. **S2**; Fig. **S3**).

Traditional morphometrics. Nine of the 12 examined meristic variables showed significant differences, of which only three differed significantly between the three groups (*H. carpintis*, *H. pantostictus* and putative hybrids): DR, PR, and SLS. The other six variables showed differences between at least two groups: DS, AS, AR, GR, SFLL,

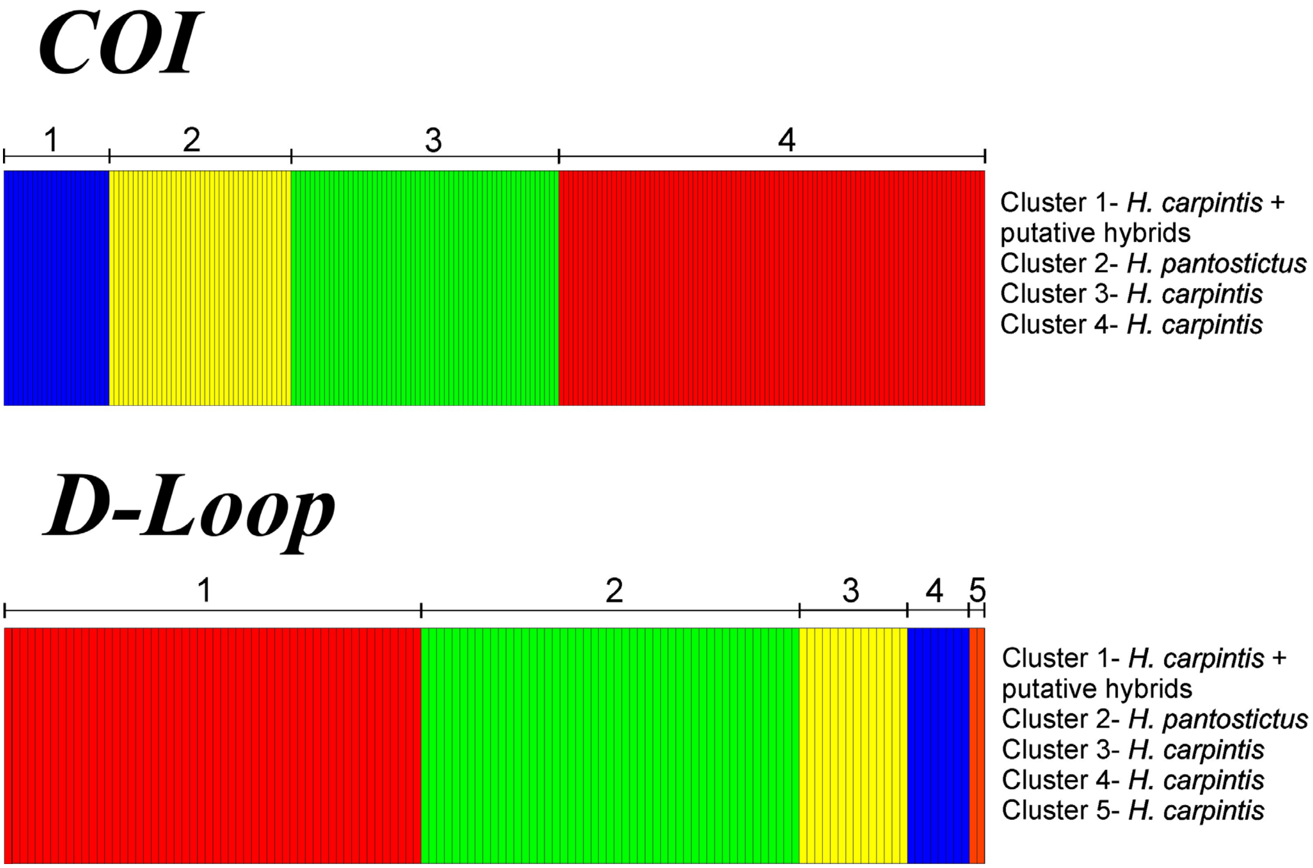

FIGURE 5 | Genetic groups recovered with the mitochondrial markers *COI* and *D-Loop*, for the populations of the species *Herichthys carpintis* and *H. pantostictus*, as well as for the populations of putative hybrids.

and TSLL. The following variables did not show differences: PFR, CS, and SSLL (Tab. 1; Fig. **S4**). On the other hand, for morphometric variables, an ANOVA adjusting for the proportions revealed that 24 of the 25 variables showed significant differences, of which 11 differed significantly between the three groups: LDF, DFE, AFE, LPF, LVF, UML, LLM, LCP, LDB, BHE, and EYD (Tabs. 2, **S5**). An ANCOVA adjusted using Mosimann's method revealed significant differences in all 27 morphometric variables, of which 19 differed significantly between the three groups (Tab. **S6**). Notably, while these analyses revealed significant differences between the three groups, the variation in the identified variables within each group exhibited a high degree of overlap, preventing the identification of diagnostic features for any group. However, the putative hybrid specimens tended to be more similar to *H. pantostictus* than to *H. carpintis*. Indeed, the PCA showed that the putative hybrids were more similar to *H. pantostictus* than to *H. carpintis*. For the meristic variables, the first two components explained 62.0% of the total variation. For the morphometric variables adjusted by proportions and Mosimann's method, the first two components explained 52.5% and 80.0% of the total variation, respectively (Fig. 6).

TABLE 1 | Descriptive statistics for the 12 meristic data used in this study for *Herichthys* species. The mode, minimum (min) and maximum (max) for each character are presented. The bold numbers represent the characters that showed significant differences between the groups.

Character	*H. carpintis*			*H. pantostictus*			Putative hybrids		
	Min	Mode	Max	Min	Mode	Max	Min	Mode	Max
Number of spines in the dorsal fin (DS)	15	16	17	14	16	18	15	16	17
Number of rays in the dorsal fin (DR)	7	**10**	12	9	**11**	13	9	**12**	12
Number of spines in the anal fin (AS)	4	5	6	4	5	6	4	5	6
Number of rays in the anal fin (AR)	4	8	10	7	9	11	8	9	10
Number of rays in the pectoral fins (PR)	10	**14**	15	13	**15**	16	12	**14**	15
Number of rays in the pelvic fins (PFR)	4	5	5	4	5	5	4	5	5
Number of gill rakers in the first arch (GR)	6	7	9	5	7	9	6	7	9
Number of scales in a longitudinal series (SLS)	21	**26**	30	16	**27**	30	25	**28**	31
Number of circumpeduncular scales (CS)	14	16	17	12	16	19	14	16	19
Number of scales in the first portion of the lateral line (SFLL)	12	18	22	8	19	22	16	19	22
Number of scales in the second portion of the lateral line (SSLL)	2	10	20	3	10	20	5	11	13
Total number of scales in the lateral line (TSLL)	14	28	32	12	29	35	19	30	34

TABLE 2 | Descriptive statistics for the 25 morphometric characters adjusted as proportions of the standard length used in this study for *Herichthys* species. The mean, minimum (min) and maximum (max) for each group are expressed as percentages. The bold numbers represent the characters that showed significant differences between the three groups.

Character	*H. carpintis*			*H. pantostictus*			Putative hybrids		
	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
Total length of the anal fin (LAF)	0.3161	0.4037	0.5427	0.2681	0.3669	0.4788	0.3246	0.3752	0.4153
Total length of the dorsal fin (LDF)	0.5174	**0.722**	0.8752	0.5488	**0.688**	0.8276	0.5735	**0.6546**	0.7247
Length of the dorsal fin of spines (DFE)	0.4073	**0.546**	0.6256	0.3681	**0.4999**	0.5911	0.4335	**0.4787**	0.5313
Length of the dorsal fin of rays (DFR)	0.0772	0.1703	0.2987	0.0901	0.1824	0.3405	0.1178	0.1734	0.2444
Length of the anal fin of spines (AFE)	0.1844	**0.2466**	0.314	0.0913	**0.1974**	0.2161	0.1644	0.2111	0.2564
Length of the anal fin of rays (AFR)	0.0735	0.1522	0.2879	0.0542	0.1653	0.2956	0.1184	0.1628	0.2123
Length of the pectoral fin (LPF)	0.1766	**0.2603**	0.3125	0.1666	**0.2373**	0.2911	0.1884	**0.227**	0.2672
Length of the pelvic fin (LVF)	0.1932	**0.2813**	0.3962	0.01913	**0.244**	0.3213	0.1857	**0.2331**	0.2888
Predorsal length (PDL)	0.1384	0.3305	0.3909	0.2584	0.3478	0.4211	0.3041	0.348	0.4135
Preanal length (PAL)	0.4965	0.6747	0.726	0.4945	0.6538	0.7314	0.602	0.6464	0.6965
Postorbital length (POL)	0.1127	0.1489	0.2095	0.1012	0.1485	0.3673	0.1442	0.163	0.3338
Length of the upper maxilla (UML)	0.0419	**0.0804**	0.1285	0.0489	**0.0759**	0.1178	0.0366	**0.0594**	0.0888
Length of the lower maxilla (LLM)	0.0419	**0.0804**	0.1285	0.0489	**0.076**	0.1178	0.0453	**0.0603**	0.0888
Length of the caudal peduncle (LCP)	0.0729	**0.1049**	0.1587	0.077	**0.1123**	0.1404	0.1028	**0.1287**	0.1552
Length of the dorsal fin at its base (LDB)	0.3536	**0.5393**	0.6444	0.236	**0.5152**	0.6828	0.4714	**0.5256**	0.5869
Length of the anal fin at its base (LAB)	0.157	0.1946	0.2544	0.124	0.1935	0.2663	0.1664	0.215	0.2541
Head length (HLE)	0.2936	0.3437	0.3095	0.1851	0.3375	0.3834	0.3119	0.342	0.3758
Snout length (SNL)	0.0688	0.109	0.1619	0.0678	0.1167	0.1639	0.0923	0.1145	0.2106
Length of the ascending premaxillary process (LPP)	0.1038	0.1484	0.2082	0.856	0.1544	0.1863	0.1242	0.151	0.1723
Length of the post ascending premaxillary process (PPP)	0.2131	0.2747	0.3329	0.1922	0.2617	0.3391	0.2411	0.2689	0.3103
Distance between the anal fin and the base of the pelvic fins (DBF)	0.2303	0.2928	0.3754	0.2208	0.2886	0.3552	0.2311	0.2843	0.3356
Body height (BHE)	0.3925	**0.456**	0.5829	0.3255	**0.4091**	0.4671	0.2825	**0.4018**	0.4539
Height of the caudal peduncle (HCP)	0.1304	0.1617	0.2185	0.1304	0.161	0.191	0.1334	0.1492	0.1697
Eye diameter (EYD)	0.0636	**0.1011**	0.1529	0.0538	**0.0851**	0.1166	0.0625	**0.0764**	0.0911
Interocular distance (IOD)	0.0947	0.1218	0.1863	0.0787	0.1155	0.1461	0.1013	0.1157	0.1406

Geometric morphometrics. The PCA indicated that the first three components explained 52% of the total variation (PC1: 20.4%, PC2: 17.9%, and PC3: 13.8%). The graph of the first two components shows an overlap in body shape between the parental species *H. carpintis* and *H. pantostictus* (Fig. 7B). While each species seems to occupy different areas in the multivariate space, the putative hybrid specimens were intermediate between both parental forms. A similar pattern was observed when components one and three were compared (Fig. 7C). However, when components two and three were compared (Fig. 7D), the shape of the putative hybrids was more similar to *H. pantostictus*. In the canonical variate analysis, a different shape was recovered for each group, although this could represent an artifact of the method (Fig. 7A).

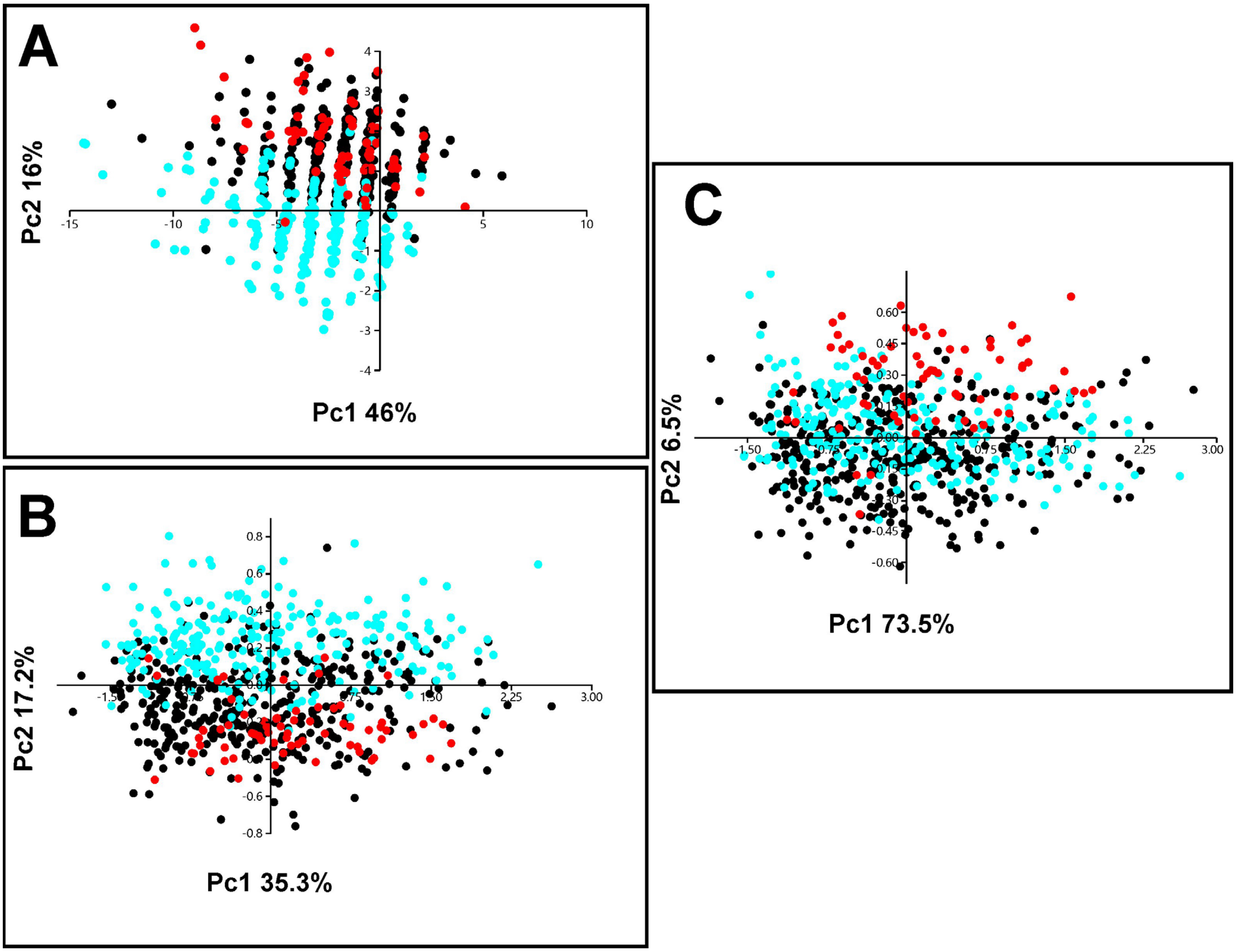

FIGURE 6 | Principal component analysis of traditional morphological characters. Meristic characters (**A**), morphometric characters adjusted by proportions (**B**) and morphometric characters adjusted by the Mosimann's method (**C**). *Herichthys carpintis* (turquoise), *H. pantostictus* (black), and putative hybrids (red).

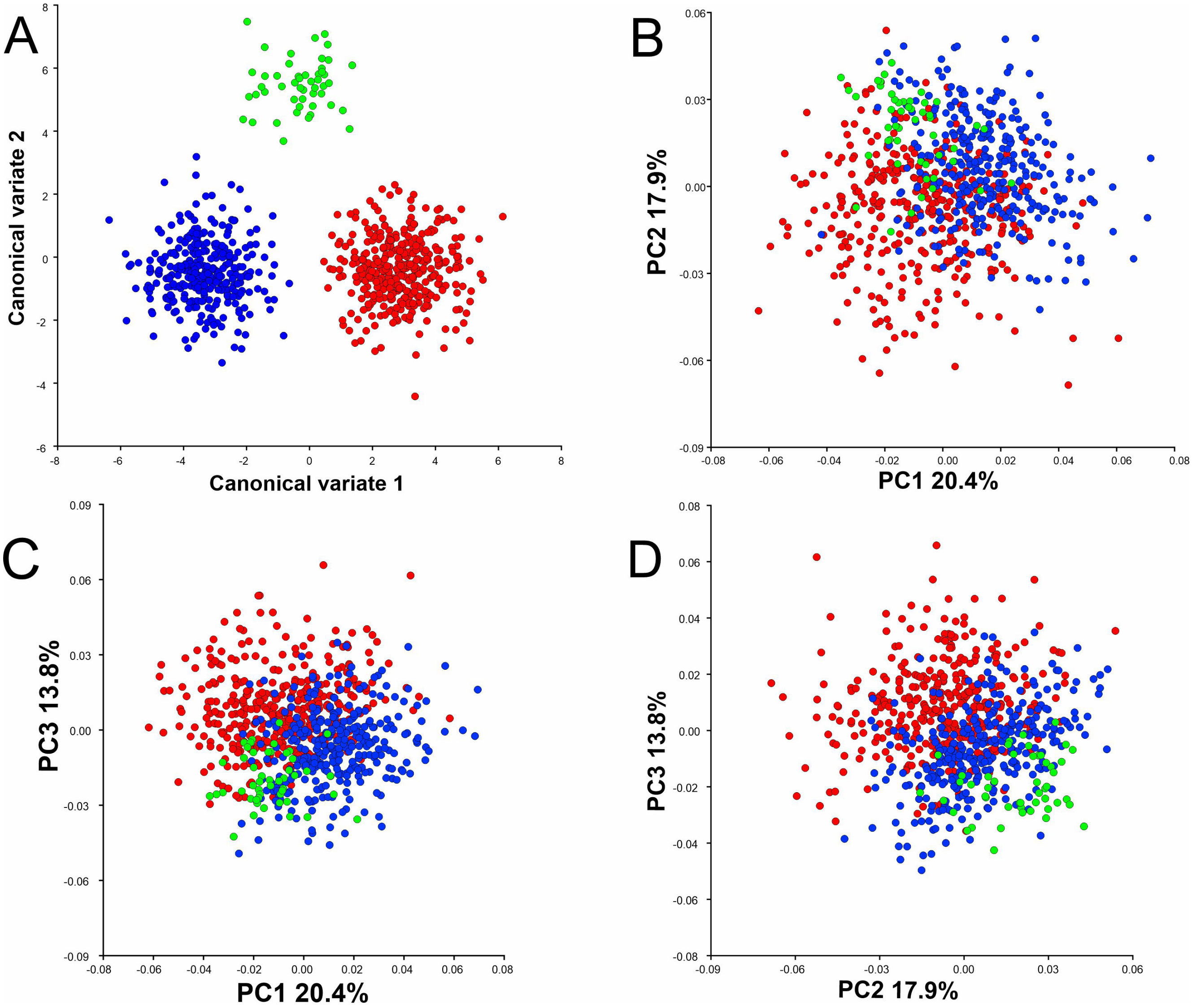

FIGURE 7 | Canonical variate analysis (**A**) and principal component (PC) analysis of the body shape. PC1 *vs*. PC2 (**B**), PC1 *vs*. PC3 (**C**) and PC2 *vs*. PC3 (**D**). *Herichthys carpintis* (red), *H. pantostictus* (blue), and putative hybrids (green).

DISCUSSION

Hybridization is common in cichlids and has been used to explain the high rate of diversification of this species (Streelmans *et al*., 2004; Albertson, Kocher, 2005; Stelkens, Seehausen, 2009; Parsons *et al*., 2011; Seehausen, Wagner, 2014; Santos-Santos *et al*., 2021), regardless of whether it is asymmetric or symmetric. Asymmetric introgression, as suggested in our study, has been reported in African cichlids (Nevado *et al*., 2011) and recently among other species of the genus *Herichthys* in northeastern Mexico (Hulsey, García de León, 2013; Magalhaes *et al*., 2015; Hulsey *et al*., 2016). Among the possible causes are a dispersion bias towards females due to the territorial behavior of the males, an asymmetric break in the prezygotic recognition systems mediated by sexual selection, and a possible molecular adaptation (Hulsey, García de León, 2013).

Firstly, female-biased dispersal has been documented in African cichlids with different parental care behaviors (Bose *et al*., 2022), either as a mechanism to avoid competition for mates or prevent inbreeding or competition for resources; the latter being the only one that can explain female-biased dispersal in monogamous species (Prugnolle, De Meeus, 2002). In the particular case of the species in the genus *Herichthys*, a mother-father family model is presented where the males are larger (Rican *et al*., 2016), which would imply obtaining and maintaining a large territory that would require the dispersal of females.

Secondly, unlike cichlids in Africa, which are distributed in lakes, most cichlids in Central America are distributed in rivers, translating into well-known differences in nuptial coloration patterns. While species show a great variety of colorations in Africa, most species show a dichromatic coloration pattern of black and white in Central America (Rican *et al*., 2016; Pérez-Miranda *et al*., 2018). Experimental evidence has revealed that African cichlids mate indiscriminately in eutrophicated environments due to nuptial coloration patterns becoming indistinguishable, eroding species diversity (Seehausen *et al*., 1997). However, in the case of *H. carpintis* and *H. pantostictus*, their sympatric populations develop in shallow rivers where visibility would not be an obstacle. Despite exhibiting sympatry in most of their distribution areas, both species have a completely different phylogeographic history (Pérez-Miranda *et al*., 2023).

Thirdly, a possible molecular adaptation could lead to asymmetric hybridization, where hybrids show better fitness than their parental species under certain selective pressures. A different mitochondrial genome can increase success in novel environments or replace one containing many deleterious mutations (Nikelski *et al*., 2023). For example, in the case of hybrids between *Herichthys cyanoguttatus* Baird & Girard, 1854, and *H. minckleyi* (Kornfield & Taylor, 1983), it has been posited that water temperature may promote the positive selection of certain amino acids in some mitochondrial proteins that facilitate adaptation to cold environments (Hulsey *et al*., 2016; Bell *et al*., 2019). In the case reported in our study, further analysis using a landscape genomics approach would be needed to evaluate the possibility of different selective pressures in both parental and hybrid populations.

Notably, the three asymmetric hybridization scenarios postulated above occur preferentially in endemic species with restricted distribution, where introgression provides new sources of genetic variation for evolution and where hybridization is relatively contemporary (Hulsey *et al*., 2016), unlike our results that suggest hybridization that occurred 1.2–1.3 Ma (Fig. 4). Therefore, we cannot discount that hybridization has been bidirectional and selection has favored only individuals carrying the mitochondrial DNA of *H. carpintis*.

It has been reported that gene flow or introgression can be temporally or spatially restricted and affect only some parts of the genome. Indeed, it has been documented that only a few generations are needed to eliminate traces of the nuclear DNA of some of the parental species (Baack, Reiseberg, 2007; Nevado *et al*., 2011). For example, in experimental crosses between *Gambusia holbrooki* Girard, 1859, and *G. affinis* Baird & Girard, 1853, within only two years, the mitochondrial DNA of *G. holbrooki* increased in frequency compared to that of *G. affinis*, whose hybrids were less efficient in perpetuating their genome (Scribner *et al*., 2000).

An alternative scenario that could explain the presence of mitochondrial DNA under the assumption of bidirectional hybridization is double uniparental inheritance, a phenomenon so far only described in bivalves. While females transmit their mitochondrial lineage to all descendants, males only transmit it to male descendants, resulting in two mitochondrial lineages in the same species (Ladoukakis, Zouros, 2017). If this were the case for *H. carpintis* and *H. pantostictus*, the male lineage should have persisted over time in the putative hybrid populations, which is unlikely, especially if females maintain the homoplasmy of the mitochondrial DNA.

A final explanation for the presence of *H. carpintis* mitochondrial DNA in individuals that exhibited the phenotype and nuclear *ITS-1* haplotypes of *H. pantostictus* is paternal leakage, where both maternal and paternal mitochondrial genomes are present in the same individual (Ladoukakis, Zouros, 2017; Mastrantonio *et al*., 2019). Paternal leakage usually leads to a heteroplasmy in the length differences of tandemly repeated sequences in the mitochondrial *D-Loop* (Brown *et al*., 1992). However, the mitochondrial *D-Loop* fragment length was identical for all individuals examined in our study. Nonetheless, we cannot rule out that heteroplasmic lineages could be eliminated either by segregation or purifying selection.

A similar model to that described in our study has been previously reported in the Poeciliidae family in Eastern Mexico. Yellow swordtail (*Xiphophorus clemenciae* Álvarez, 1959) is a species with a swordtail phenotype whose mitochondrial haplotypes belong to the platyfish (*X. maculatus* Günther, 1866). Analyses of mitochondrial and nuclear genes revealed that this species likely originated through hybridization between female platyfish (*X. maculatus*) and male swordtail (*X. helleri* Heckel, 1848) approximately 1 Ma (Meyer *et al*., 2006; Kang *et al*., 2013), a similar temporal frame to the hybridization inferred between *Herichthys* species.

In our study, the results of the morphological analysis of both traditional features and geometric morphometrics did not allow the definitive differentiation of the putative hybrids from the parental species. However, some meristic features such as DR and SLS, as well as morphometric features associated with the dorsal fin and the caudal peduncle, could help to discriminate them (Tabs. 1–2; Figs. 6–7, **S7**), as has been suggested in other studies were hybrids develop a completely different phenotype to their parental species (Albertson, Kocher, 2005; Stelkens, Seehausen, 2009; Parsons *et al*., 2011; Seehausen, Wagner, 2014; Santos-Santos *et al*., 2021). However, as has been previously documented in other species of the genus, a high degree of overlap exists in the ranges of variation of meristic and morphometric features, making them unsuitable for use in diagnostics (Pérez-Miranda *et al*., 2018). Only the canonical variate analysis of geometric morphometrics recovered three discrete groups, but this could be due to the nature of the discriminant function. Therefore, despite these results, we suggest that putative hybrids present a different shape due to the broad variation observed in other species of this genus (Mejía *et al*., 2015; Pérez-Miranda *et al*., 2018). However, the PCA results showed a slight segregation in the shape of the putative hybrids, potentially suggesting morphological differentiation, as has been recently documented in another group of neotropical cichlids (Olave *et al*., 2022), although those populations showed a restricted distribution in lentic environments.

In summary, our results show that the mitochondrial haplotypes of putative hybrids identified as *H. pantostictus* from the Santa María River were recovered as a monophyletic group nested within the haplotypes of *H. carpintis*. In contrast, their nuclear *ITS-1* haplotypes were recovered nested within the rest of the *H. pantostictus* haplotypes. The high divergence time of the parental species (11–12 Ma) allows us to rule out maintenance of ancestral polymorphism, although recent studies indicate that it may contribute to the diversification of other cichlids (Olave *et al*., 2022; Astudillo-Clavijo *et al*., 2023). The low nuclear divergence and the discordance between the mitochondrial and nuclear phylogenetic trees suggest an ancient hybridization event between these two species. While several processes could have led to the hybridization (Meyer *et al*., 2006; Hulsey *et al*., 2016; Hata *et al*., 2019), the actual causes cannot be determined at present. Further studies evaluating the level of genetic introgression and crossbreeding experiments between both parental species under laboratory conditions are needed to understand the role of hybridization in the diversification of this genus.

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AUTHORS' CONTRIBUTION

Omar Mejía: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing-original draft.

Fabian Pérez-Miranda: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing-original draft, Writing-review and editing.

Amairany Bernal-Portillo: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing-original draft.

ETHICAL STATEMENT

All material used in this study proceeds from museum specimens, no collection permit was required.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

COMPETING INTERESTS

The authors declare no competing interests.

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