PRIMARY RESEARCH PAPER

Astyanax caucanus: microsatellite *loci* development and population genetics in the Cauca River, Colombia

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Abstract *Astyanax caucanus* is an endemic fish species to the Magdalena-Cauca basin in Colombia. It is considered a Least Concern species by the International Union for Conservation of Nature, and currently, it is not a fishery resource. Its fertilized eggs may drift up to 4–5 days before hatching and can be carried up to 340 km given the water velocity of the river. Although *A. caucanus* is listed as short -migratory species (< 50 km), this study hypothesized that it exhibits gene flow along the middle and lower section of the Cauca River due to the great potential for larval dispersal. To test this hypothesis, we developed a set of species-specific microsatellite primers suitable for population genetic studies. Genetic structure analyses with 193 samples evidenced two genetic stocks that coexist, comigrate, and exhibit gene flow along the study area. Both stocks show high genetic diversity indices (Na and H_E) and effective population sizes ($N_e > 1000$), but also show evidence of bottlenecked populations and high values of the inbreeding coefficient (F_{IS}). Finally, these results are useful to understand the effects of other anthropic activities, besides fishing pressure on population bottlenecks found for other fish species cohabiting the area.

Keywords Gene flow · Genetic diversity · Molecular markers · Population bottleneck · Neotropical fish

Introduction

Astyanax, one of the 142 valid genera family Characidae (Fricke et al., 2023a), is widely distributed in the Neotropical region, from southern United States to northern Argentina (Nelson et al., 2016). These fishes commonly known as tetras are highly diverse, comprising more than 129 species (Fricke et al., 2023b) that exhibit diagnostic characters generalized within the family, which hampers the taxonomic classification based on their morphology (Mirande, 2019). The final phylogenetic arrangement of the genus remains to be elucidated, although many studies have aimed to disentangle the phylogenetic relationships of *Astyanax* (Ornelas-García et al., 2008; Mirande, 2009, 2019; Mello et al., 2015; Casane & Rétaux, 2016; Rossini et al., 2016; Schmitter-Soto, 2016, 2017; Piscor et al., 2019; Terán et al., 2020). Complementary information about morphology and DNA sequences has allowed to validate the identity of some *Astyanax* species and assign others to valid or resurrected genera (Terán et al., 2020); however, such information is absent for other members of this

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genus limiting their validation or redescription (Terán et al., 2020). This is the case for *Astyanax caucanus* (Steindachner, 1879), an endemic fish species to the Magdalena-Cauca hydrographic region in Colombia, often confused with *A. magdalenae* Eigenmann & Henn, 1916 because their similar morphology and shared geographic distribution (Ruiz-C. et al., 2011; García-Alzate et al., 2021).

Astyanax caucanus is an omnivorous species that plays an important ecological role for the Magdalena-Cauca River basin (Gutiérrez-Moreno & de la Parra-Guerra, 2021). Due to its small size and high population number, *A. caucanus* among other species is considered an important part of the diet for larger species, not only fishes but also birds, turtles, and otters (Gutiérrez-Moreno & de la Parra-Guerra, 2021). This is a potamodromous and short-distance migratory species (<50 km) with early maturity and periodic reproduction (Jiménez-Segura et al., 2021). *Astyanax caucanus* is included in the IUCN red list as a least concern (LC) species, due to its wide distribution and that there are no major threats for its populations (Villa-Navarro & Sanchez-Duarte, 2014). However, the genetic status of this species remains unknown, although their natural habitat is constantly exposed to anthropic impacts due to the Magdalena-Cauca River basin, Colombia's primary developmental area, housing 77% of its inhabitants and contributing to 80% of its Gross Domestic Product (Restrepo et al., 2021). This has resulted in a significant anthropic impact on aquatic biodiversity in the region, including sewage discharge, irresponsible mining, the desiccation of floodplain lakes for livestock and agricultural expansion, and dam construction, among other factors (Angarita et al., 2021; Gutiérrez-Moreno & de la Parra-Guerra, 2021). In February 2019, the closure of the gates during an emergency at the Ituango dam construction on the Cauca River's main channel caused the complete disruption of the river's flow, resulting in the death of thousands of low-migration-range fish located 10 km downstream of the dam. Consequently, it is essential to generate information about the structure and genetic diversity of the populations of *A. caucanus* to estimate the risk category and design conservation strategies for the species.

Colombia lacks population genetic studies for *Astyanax* species, in contrast to Mexico (Strecker et al., 2003; Panaram & Borowsky, 2005; Hausdorf et al., 2011; Bradic et al., 2012; Herman et al., 2018;

Pérez-Rodríguez et al., 2021) and Brazil (Leuzzi et al., 2004; Peres et al., 2005; Sofia et al., 2006; Ferreira et al., 2016; Freitas-Lidani et al., 2018; Limeira et al., 2019). In Mexico, the studies are focused mainly on analyzing surface versus cave populations using different molecular markers that include microsatellite *loci* (Strecker et al., 2003; Panaram & Borowsky, 2005; Hausdorf et al., 2011; Bradic et al., 2012; Pérez-Rodríguez et al., 2021). In Brazil, different molecular approaches such as RAPDs (Leuzzi et al., 2004; Sofia et al., 2006; Ferreira et al., 2016; Freitas-Lidani et al., 2018), allozymes (Peres et al., 2005), and microsatellite *loci* (Ferreira et al., 2016; Limeira et al., 2019) are used to analyze the diversity and structure of *Astyanax* populations. In terms of genetic diversity, these studies show that high allelic richness and heterozygosity is generalized within the genus. So far, only one study found evidence of gene flow among cave and surface populations in geographically close locations (Panaram & Borowsky, 2005), whereas other studies reveal that *Astyanax* populations are frequently structured and may exhibit structure in population separated by only 5 km (Sofia et al., 2006). These works suggest that such structure may be related to an isolation by distance model (Strecker et al., 2003; Leuzzi et al., 2004; Peres et al., 2005; Sofia et al., 2006; Ferreira et al., 2016; Freitas-Lidani et al., 2018; Garita-Alvarado et al., 2021; Pérez-Rodríguez et al., 2021), phylogenetic distance (Hausdorf et al., 2011; Garita-Alvarado et al., 2021), or assortative mating (Bradic et al., 2012). Based on the coancestry analysis, Ferreira et al. (2016) and Garita-Alvarado et al. (2021) show the coexistence of different genetic groups of *Astyanax* in the same studied localities.

Aiming to generate information useful for developing management and conservation policies for this species, this study assessed the genetic status and tested the hypothesis that *A. caucanus* exhibit gene flow along middle and lower sections of the Cauca River. If *A. caucanus* fish fry exhibits a similar behavior as some congeners that become free swimming and ready to feed on plankton at 4–5 days post fertilization (Axelrod et al., 1971; Hinaux et al., 2011; Stevanato & Ostrensky, 2018; dos Santos et al., 2020), in the absence of physical barriers, it is expected that this short-distance migrator may exhibit gene flow in an area of 325 km. Since the annual average water velocity in the area

is 1.18 ± 0.52 m/s (middle section: S2-S3; Fig. 1) and 0.50 ± 0.18 m/s (lower section: S4-S8) (unpublished data), the larvae may drift up to 340 km along the main channel of the river until going into the floodplain lakes. To test this hypothesis, this work developed a set of species-specific microsatellite *loci* in population genetic studies of *A. caucanus*, which were able to measure the genetic diversity of this tetra fish in the middle and lower sections of the Cauca River.

Materials and methods

Sample collection and DNA extraction

For population genetic purposes, this study used 193 ethanol-preserved muscle or tail tissues from individuals of *A. caucanus* collected in the middle and lower basin of the Cauca River to extract genomic DNA. The tissues were collected during the same fish rise period (*subienda; December–March*) in 2019 and 2020 by Grupo de Ictiología de la Universidad de Antioquia (GIUA). The specimens were collected along the main channel of the river in different environments including small affluents (middle basin) and floodplain lakes (middle and lower basin). The collection area was divided into nine sections, S1 to S8 as previously described by Landínez-García & Márquez (2016) and PHI corresponding to the current site of the Ituango hydropower project reservoir. Individuals were caught in seven of these sections; S1 and PHI were excluded due to lack of samples despite collection efforts. Sample number per site was as follows: S2/3: 30, S4: 31; S5: 47, S6: 37; S7/8: 48 (Fig. 1). Due to the extremely low number of individuals in those sections, S2 was merged with S3 (mouth of the Espiritu Santo River on the east margin of the Cauca River), and S7 (floodplain lakes located near the mouth of the Cauca River in the Magdalena River) with S8 (main channel of the Cauca River near the mouth of the Cauca River in the Magdalena River). Extraction of DNA was carried out using the PureLink™ Genomic DNA Mini Kit (Invitrogen), following manufacturer's instructions and performing overnight digestions.

Phylogenetic analysis

To avoid confusions with other phylogenetically related species of *Astyanax* cohabiting the area, the mitochondrial gene *cytochrome c oxidase* subunit *1* (MT-CO1) of 209 samples was partially sequenced using a four primers mix: VF2_t1 5′-TCAACCAAC CACAAAGACATTGGCAC-3′, FishF2_t1 5′-TCG ACTAATCATAAAGATATCGGCAC-3′, FishR2_t1 5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′ (Ward et al., 2005) and FR1d_t1 5′-CACCTCAGG GTGTCCGAARAAYCARAA-3′ (Ivanova et al., 2007). Sequencing included samples from individuals tentatively identified as *A. caucanus, Astyanax* aff. *fasciatus,* and *Astyanax microlepis* Eigenmann, 1913 that were identified through traditional morphometrics following original descriptions of species (Steindachner, 1879; Eigenmann, 1913; Eigenmann & Henn, 1916); samples of *A. caucanus* collected in the type locality (Tarazá River), and *A.* aff. *fasciatus* (Colosó-Sucre: IAvH-CT-17785, Cimitarra- Santander: IAvH-CT-24679; Barrancabermeja- Santander: IAvH-CT-24548), were included as reference for validated nominal species. The amplification was performed following Rangel-Medrano et al. (2020) with some modifications to the final concentrations of DNA and primers: 1.0–1.5 ng/μL of DNA and 0.2 pmol/μL of the four-primer mix. The thermal profile for PCR comprised an initial denaturation of DNA at 90 °C for 3 min, followed by 35 temperature cycles of denaturing step at 90 °C for 30 s, annealing step at 56 °C for 60 s, and extension step at 72 °C for 30 s, and a final extension step at 72 °C for 2 min.

Sequences were purified, cut, aligned, and translated into amino acids to confirm absence of stop codons using GeneiousPrime® v2022.1.1 (www. geneious.com), and the software DnaSP v6.12.03 was used to obtain the list of haplotypes for further analyses. All the new sequences for *A. caucanus* and other species generated in this study are available on GenBank (accession numbers: OR064539—OR064743). Partial sequences MT-CO1 of *A. microlepis* (UdeA; BOLD: CIUA979), *Psalidodon fasciatus* (Cuvier, 1819) from Venezuela (GenBank KY267682), *Psalidodon fasciatus* from Brazil (GenBank KY268274), and the outgroup *Brycon henni* Eigenmann, 1913 (GenBank KP027535) were also included for phylogenetic tree reconstruction (Fig. 2). The software jModelTest v2.1.10 (Darriba et al., 2012) was used to
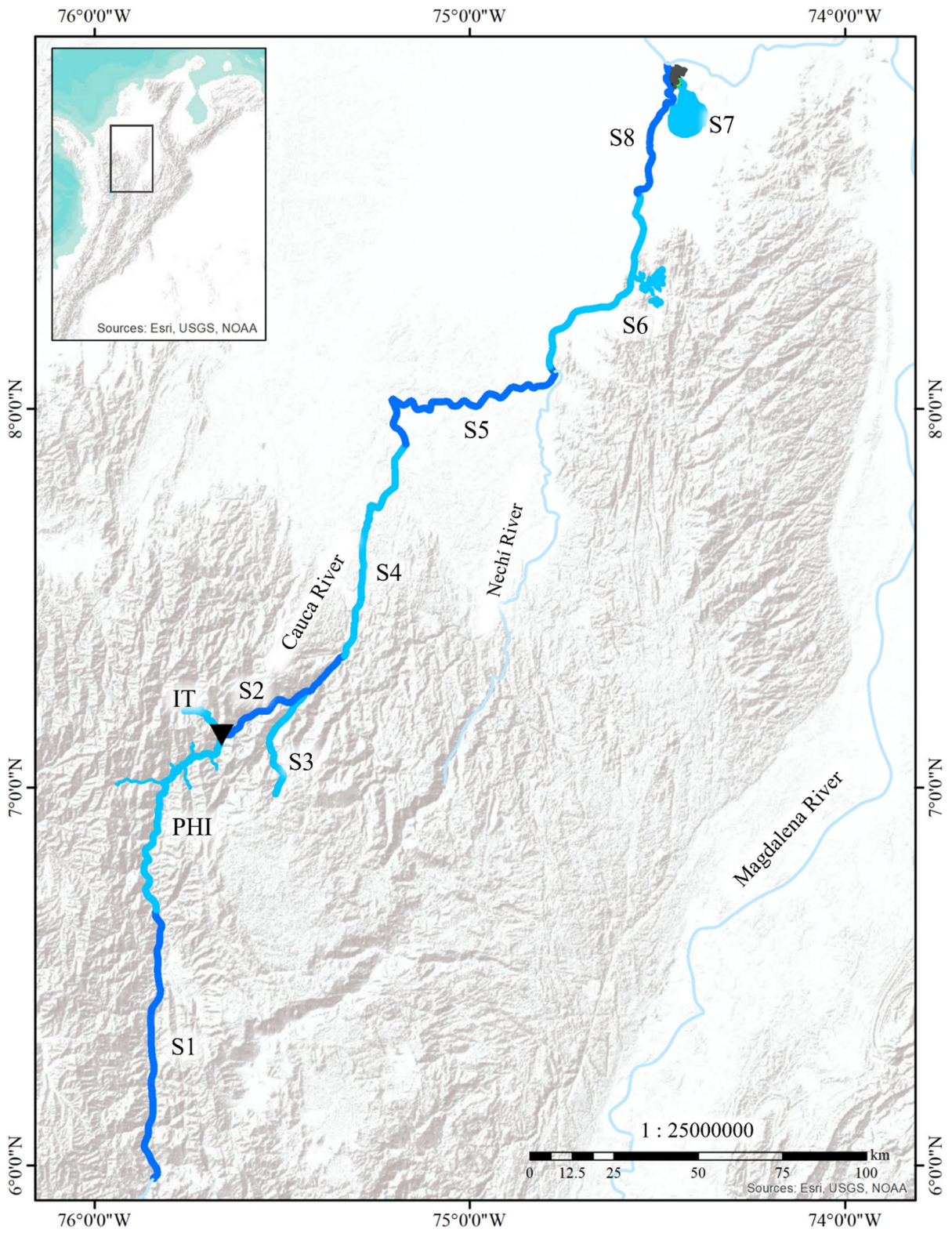
select the best-fit nucleotide substitution model of the complete dataset. The tree was constructed using the Beauti v2.6.7 application of Beast2 (Bouckaert et al., 2014) with the following parameters for the run: HKY + G; Gamma category count: 4; 400,000,000 MCMC; 10% as burn-in; and storeEvery 40,000. The other parameters of the run were left as default. The run was carried out using the CIPRES platform (https://www.phylo.org/). Then, convergence of parameters (ESS > 200) in the run was checked with Tracer v1.7.1 application of Beast2. To summarize the 10,000 trees, we used TreeAnnotator v2.6.0 application of Beast2; finally, FigTree v1.4.4 application was used to visualize the final tree.

Microsatellite loci development

Aiming to identify microsatellite *loci* and develop a species-specific set of primers, the shotgun genomic library from one individual of the most frequent haplotype was sequenced with the Miseq Illumina platform. The subsequent analysis of the genomic data was performed as described previously by Landínez-García & Márquez (2016, 2018). Briefly, the reads with less than 50 bp, low-quality regions or duplicated were eliminated from analysis using Prinseq-lite v0.20.4 (Schmieder & Edwards, 2011), the cleaned reads were analyzed with PAL_FINDER v0.02.03 to extract potentially amplifiable *loci* (PAL), the primers were designed using PRIMER3 v.2.0 (Rozen & Skaletsky, 2000) and the correct primer alignment was verified by electronic PCR (Rotmistrovsky et al., 2004).

A set of 30 microsatellite *loci* were tested experimentally to check amplification consistency and level of polymorphism in 30 random samples. The 5′end of the forward primers was attached covalently to adapters as described in Table 1 complementary to a sequence fluorescently labeled with 6-FAM, VIC, NED, and PET (Blacket et al., 2012; Table 1). Concentrations and conditions for PCR amplifications were as proposed by Landínez-García & Márquez (2018) with some modifications described hereunder.

The following final concentrations were used for the 10 μL amplification reactions: 5–10 ng/μL of DNA, 1 X of Platinum Mix and 2.5% of Enhancer GC, both from the Platinum™ Multiplex PCR Master Mix kit (Invitrogen), 0.50 pmol/μL of each forward primer, 1 pmol/μL reverse primer, and 0.50 pmol/μL of the universal-labeled primer. Additionally, amplifications were carried out in a T100 thermal cycler (BioRad) using the following profile that did not include both, elongation step and final extension: 3 min at 94 °C for initial denaturation, 35 cycles consisting of 35 s at 90 °C for denaturation step, and 35 s at 56 °C for annealing step. Finally, amplicons were separated using a SeqStudio Genetic Analyzer (Applied Biosystems) and LIZ600® (Applied Biosystems) as internal molecular size marker. The size of each fragment was annotated using the software GeneMarker v3.0.0 (SoftGenetics LLC®). Those microsatellite *loci* that satisfied the selection criteria described by Landínez-García & Márquez (2016, 2018) (polymorphism level, band resolution, specificity, desired fragment size, and heterozygosity) were used for further population genetic analyses.

Population genetics

The genetic study included 193 samples of *A. caucanus* genotyped using 12 primer pairs selected as described above. Micro-Checker v2.2.3 (van Oosterhout et al., 2004) was run to detect potential genotyping errors. The software GenAlEx v6.503 (Peakall & Smouse, 2012) was used to measure the average number of alleles per *locus* and the software Arlequin v3.5.2.2 (Excoffier & Lischer, 2010) to estimate the observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient (F_{IS}), and departures of Hardy–Weinberg (HWE) and Linkage (LE) equilibria. In addition, we used Cervus v3.0 (Kalinowski et al., 2007) to calculate the polymorphic information content (PIC) for each microsatellite *locus*. For all analyses with multiple comparisons, sequential Bonferroni correction (Rice, 1989) was applied to adjust for statistical significance. Additionally, BayeScan v2.01 (Foll & Gaggiotti, 2008) with default parameters was used to explore nonneutral selection acting on microsatellite *loci*. *Loci* were ranked according to their estimated posterior probability or posterior odds (equivalent to Bayes factor), a statistical criterion to test the model (Foll, 2012). Positive alpha values
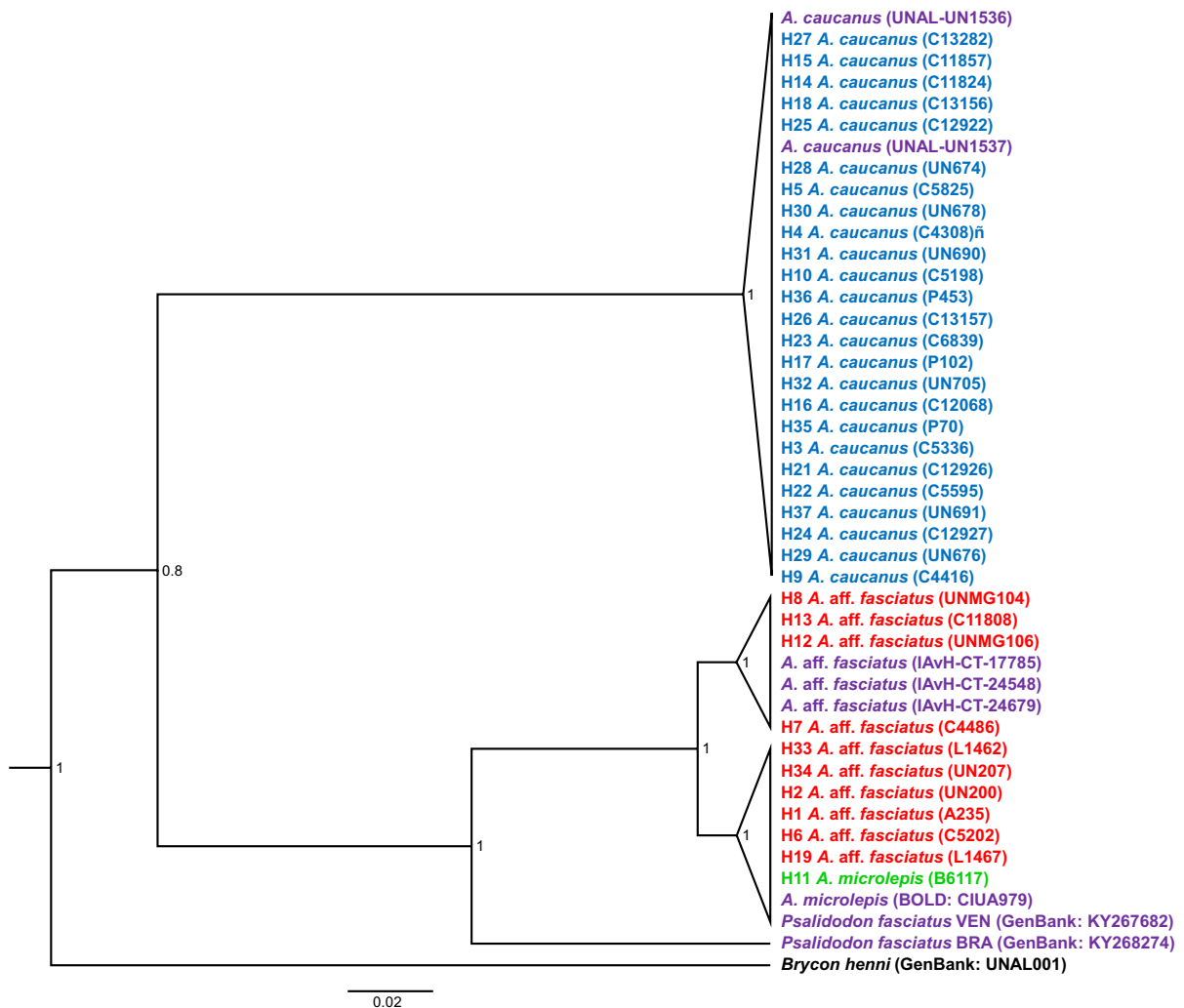
Fig. 2 Phylogenetic relationships between haplotypes in this study and some *Astyanax* species. Names in blue, red, and green represent individuals identified, through traditional mor- phometrics, as *Astyanax caucanus*, *Astyanax* aff. *fasciatus* and *Astyanax microlepis*, respectively. Names in purple represent sequences used as reference

were then used to distinguish microsatellites under diversifying selection, while negative alpha values were used to detect balancing selection.

Exploration of recent genetic bottlenecks was performed calculating the levels of heterozygosity using the Wilcoxon sign-rank test (Luikart & Cornuet, 1998) with Bottleneck v1.2.02 (Piry & Luikart, 1999) and M-ratio using Arlequin v3.5.2.2 (Excoffier & Lischer, 2010). Values of M-ratio smaller than 0.680 indicates that the population experienced recent reductions in the population size (Garza & Williamson, 2001). The software Migrate v5.0.4 (Beerli & Palczewski, 2010) was used to estimate

effective population size (N_e), setting the following parameters: Brownian model as it is recommended for Microsatellite data, θ: 0–120 (uniform), heating: 1.0, 1.5, 3.0, 1,000,000 (static), long chains: 6, number recorded steps: 20,000, number sampling increment: 200, and burn-in: 2000 for every chain. The runs was performed in CIPRES platform (https://www.phylo.org/). To transform the θ parameter in N_e, $\theta = 4 \times N_e \times \mu$ (Gilbert & Whitlock, 2015), where $\mu = 5.56 \times 10^{-4}$ changes/locus/generation (Yue et al., 2007).

The Geneclass2 software (Piry et al., 2004) was employed to assess migration events between

Table 1 Properties of 16 microsatellite loci developed for *Astyanax caucanus*

Locus	Motif	Forward–reverse primer sequences (5'—3')	Ra	Na	A_R	PIC	Adapter
Aca14	$(AAAG)_n$	F: CAGGACCATGACACAAATGG R: CCAGTTATTTCCAGCCAGTCC	201–309	28	27.990	0.965	Tail B
Aca07	$(ATT)_n$	F: TCTAAGCAGTGCAGAAAGGGG R: CACCCTATCAACCACTTGGG	122–227	32	31.356	0.959	Tail A
Aca12	$(ATCT)_n$	F: TATCACCCATCTGTTTGCCG R: GACCGAGGGTTAGACAATCAGG	207–335	29	28.954	0.958	Tail B
Aca17	$(AGTCT)_n$	F: AAAGACTCCTCCCACCCTGG R: TCCCCTCAGGCTACCTTTCC	240–335	19	18.990	0.957	Tail D
Aca25	$(ATCT)_n$	F: GGAGGTTGCAGGAAGGAGG R: AATGTGGGTGAAAGAAATTGTCC	147–279	28	27.996	0.956	Tail C
Aca06	$(ATT)_n$	F: ACACTGATCGTTCATGTTTGG R: GGCTGCAGTACAGCATTGG	211–283	24	23.976	0.952	Tail A
Aca18	$(ATT)_n$	F: GATTGGATGTCGTCTCTGCC R: CAGCAAAATGCACAAGTTTGG	249–318	23	22.987	0.947	Tail D
Aca15	$(ATCT)_n$	F: AATTGAAACAAAAGCTGGGG R: AAAACATTTTGTGAGAAGGTGC	174–306	23	22.922	0.942	Tail A
Aca16	$(ATCT)_n$	F: GTGGAGGTACAAACCCTCGG R: TTGCGATAAATGTTATTGTTGGC	164–304	24	23.988	0.942	Tail B
Aca01	$(AAAT)_n$	F: TTTATTTACAGGAATGACAGTACAGC R: GTCCAGAGAGGATTTGCTGG	140–208	17	16.983	0.931	Tail A
Aca27	$(ATCT)_n$	F: AGGGCATTGGGTTTTCAGG R: CGCCTTCCAATACAGTGTGC	195–247	12	11.975	0.911	Tail C
Aca29	$(ATTAC)_n$	F: TTGGACAAAAGTGTCCGCC R: AACTGAGTCCCTAAGTTCAGTTGC	143–173	7	7.000	0.887	Tail C
Aca22	$(ATTAC)_n$	F: TTTGGATAAAATCGTCCGCC R: CATGAAGATTGGCTTGGACG	143–173	7	7.000	0.849	Tail D
Aca21	$(ATCT)_n$	F: TGGCAAAGCTGAAGAAATGG R: ACTACTCCCTTGCCTTGCCC	126–294	19	19.000	0.805	Tail D
Aca09	$(TCCTG)_n$	F: CCAGTGGAAAACTGGAAAGCC R: TTGCAACAGCTAAAGCAGGG	262–287	6	6.000	0.758	Tail B
Aca24	$(ATTAC)_n$	F: TGTTTGTGCTGGAAGGTTGC R: CCCTCTGACCTCCTTTAGGTCC	195–285	5	4.987	0.754	Tail C
Accross loci			122–335	18.938	18.050	0.904	

Ra: allelic size range; Na: number of alleles per locus; A_R: allelic richness; PIC: polymorphic information content; F and R: primer forward and reverse sequences; Tail A: GCCTCCCTCGCGCCA; Tail B: GCCTTGCCAGCCCGC; Tail C: CAGGACCAGGCTACCGTG; Tail D: CGGAGAGCCGAGAGGTG (Blacket et al., 2012)

different sites, encompassing both the overall sample and each distinct stock. The approach entailed assigning each individual to its most probable section of origin based on the collected samples. This assignment was performed using a Bayesian criterion, specifically the maximum likelihood estimator L_home/L_max, which represents the relationship between an individual's maximum likelihood of originating from the sampled section and the maximum likelihood across all sections. In addition, a statistical Monte Carlo resampling method (Paetkau et al., 2004) was applied, setting the simulation of 10,000 individuals and an error probability of 0.01.

Spatial structure of *A. caucanus* was calculated with an analysis of molecular variance (AMOVA) with the standardized statistics F'_{ST} (Meirmans, 2006) and Jost's D'est (Jost, 2008), and setting 10,000 permutations, all included in GenAlEx v6.503 (Peakall & Smouse, 2012). To test if there is correlation of genetic distance and geographical distance a Mantel test included in GenAlEx v6.503 (Peakall & Smouse, 2012) was performed

with 9999 permutations. In addition, structure was explored with a discriminant analysis of principal components (DAPC) with the R package *adegenet* (Jombart, 2008) and using the diploid genotypes of 12 *loci* (24 variables) in 193 individuals. Furthermore, genetic structure was explored in R-based Parallel Structure (implemented from Structure v2.3.4; Pritchard et al., 2000) in CIPRES (https://www.phylo.org/) using 1,000,000 MCMC and an additional 10% as burn-in; admixture and non-admixture assumptions, and the LocPrior option. Each number of clusters (K) simulated ranged from K=1 to 8 and each was simulated 20 times. After runs, the best K value was selected using the methods described by Evanno et al. (2005) and Puechmaille (2016) in the web-based software StructureSelector (Li & Liu, 2018). The coancestry plot was generated using the program Clumpak (Kopelman et al., 2015) included in StructureSelector.

Results

Phylogenetic analysis

After edition and alignment, 193 sequences of 492 bp of length (37 haplotypes) showed no start or stop codons after translation, suggesting that they are part of a functional gene. The phylogenetic tree showed three main groups (Fig. 2): (1) samples from the middle and lower Cauca River [25 haplotypes] clustered with *A. caucanus* from Tarazá. This haplogroup was considered as *A. caucanus* due to inclusion of topotype sequences of this nominal species, having priority over remaining names used to identify samples analyzed here; (2) samples of *A.* aff. *fasciatus* [4 haplotypes] clustered with samples of *A.* aff. *fasciatus* from Colosó—Sucre (IAvH-CT-17785), Cimitarra—Santander (IAvH-CT-24679) and Barrancabermeja–Santander (IAvH-CT-24548); and (3) samples of *A.* aff. *fasciatus* [6] and *A. microlepis* [1 haplotype] clustered with *A. microlepis* from the Porce River (BOLD: CIUA979), and *Psalidodon fasciatus* from Venezuela (GenBank: KY267682). All samples named *A.* aff. *fasciatus* were phylogenetically different from *Psalidodon fasciatus* from Brazil (GenBank KY268274).

Microsatellite loci development

The analysis of genomic sequencing of one individual showed that 76,206 of the total reads contained microsatellite *loci*, and the 5-mer and 6-mer were the most common repeat motifs with 42.72% and 32.25%, respectively, followed by 3-mer (10.81%), 2-mer (7.12%), and 4-mer (7.10%). Of the total microsatellite *loci*, 55,119 were potentially amplifiable, and 24,967 were validated through electronic PCR. Sixteen of the 30 *loci* tested experimentally satisfied the selection criteria described above (Table 1), amplified in the expected size range (122–335) and were highly polymorphic (average PIC: 0.904). All *loci* showed significant departures from Hardy–Weinberg equilibrium except in a few cases in the genetic stocks and four showed linkage disequilibrium (Aca01–Aca15 and Aca06Aca16) in the subgroup of 30 individuals. Both the average number of alleles per *locus* (Na) and the allelic richness (A_R) showed values greater than 18 (Table 2).

Population genetics

Four *loci* were excluded from population genetic analyses since they showed outlier data according to BayeScan v2.01 (Aca07, Aca14, Aca21; Supplementary Table 1) or showed inconsistent amplification (Aca16; Table 2). There is no evidence of scoring errors detected by Micro-Checker; additionally, all loci showed evidence for a null allele and the overall sample showed departures from Hardy–Weinberg equilibrium and significant heterozygosity deficit. According to the different analyses of genetic differentiation, *A. caucanus* does not exhibit geographic structure due to the AMOVA was not statistically significant, which agrees with the values of F'_{ST} and Jost's D'est that were low and non-significant for all sections of the river under analysis (Table 3). The Mantel test showed a weak and statistically non-significant signal of spatial correlation of genetic distances ($R^2 = 0.055$; P = 0.291). Similarly, DAPC indicated that all geographical demes constitute only one group (Fig. 3A). Furthermore, the analysis in Structure showed that all demes exhibit gene flow, although it was clear the existence of two genetic stocks (K=2) distributed along the study area (Fig. 3B).

The diversity of genetic stocks was similar to the pooled sample (Table 4) particularly, Stock1 and

Table 2 Genetic diversity with 16 microsatellite *loci* of *Astyanax caucanus* in the sampling area (Cauca) and two genetic stocks (Stock1 and Stock2) found in this study

Locus	Aca01	Aca06	Aca07	Aca09	Aca12	Aca14	Aca15	Aca16	Aca17	Aca18	Aca21	Aca22	Aca24	Aca25	Aca27	Aca29	Across *loci*
Cauca																	
Na	17	24	32	6	29	28	23	24	19	23	19	7	5	28	12	7	18.938
A_R	16.983	23.976	31.356	6.000	28.954	27.990	22.922	23.988	18.990	22.987	19.000	7.000	4.987	27.996	11.975	7.000	18.882
H_E	0.886	0.928	0.944	0.593	0.944	0.957	0.894	0.918	0.932	0.915	0.695	0.735	0.616	0.944	0.870	0.800	0.848
H_O	0.713	0.556	0.910	0.303	0.437	0.827	0.285	0.148	0.396	0.429	0.060	0.314	0.452	0.292	0.722	0.346	0.449
P_{HWE}	**0.000**	**0.000**	**0.022**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	-
F_{IS}	0.198	0.403	0.039	0.491	0.540	0.138	0.683	0.840	0.577	0.533	0.914	0.575	0.269	0.692	0.172	0.569	0.330
P_{FIS}	**0.000**	**0.000**	**0.024**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Stock1																	
Na	17.000	24.000	29.000	6.000	26.000	27.000	18.000	24.000	19.000	20.000	18.000	7.000	5.000	25.000	11.000	6.000	17.625
A_R	15.648	21.824	24.180	5.781	23.597	26.313	16.028	22.003	17.926	18.088	16.010	6.928	4.295	23.351	10.694	6.000	16.167
H_E	0.865	0.932	0.940	0.565	0.927	0.956	0.883	0.913	0.928	0.905	0.705	0.743	0.591	0.945	0.862	0.776	0.840
H_O	0.667	0.590	0.911	0.317	0.473	0.883	0.310	0.155	0.418	0.424	0.055	0.358	0.423	0.288	0.680	0.407	0.460
P_{HWE}	**0.000**	**0.000**	0.116	**0.000**	**0.000**	**0.039**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	-
F_{IS}	0.233	0.370	0.035	0.442	0.493	0.080	0.652	0.831	0.552	0.535	0.923	0.522	0.289	0.697	0.215	0.480	0.307
P_{FIS}	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Stock2																	
Na	15.000	17.000	26.000	4.000	21.000	28.000	18.000	19.000	18.000	18.000	11.000	6.000	5.000	23.000	11.000	7.000	15.438
A_R	14.428	16.940	25.216	4.000	20.592	27.653	17.837	18.851	4.862	17.912	11.000	5.859	17.959	22.905	10.843	6.843	15.231
H_E	0.893	0.898	0.946	0.579	0.919	0.946	0.894	0.909	0.929	0.870	0.667	0.695	0.634	0.928	0.873	0.729	0.832
H_O	0.800	0.492	0.908	0.277	0.371	0.723	0.237	0.133	0.354	0.439	0.071	0.231	0.508	0.300	0.800	0.231	0.430
P_{HWE}	0.050	**0.000**	**0.002**	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.018	**0.000**	0.038	**0.000**	-
F_{IS}	0.112	0.458	0.048	0.527	0.602	0.243	0.739	0.856	0.624	0.502	0.895	0.672	0.207	0.681	0.091	0.687	0.351
P_{FIS}	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

Values in bold denote statistical significance (alpha = 0.050). Na: number of alleles per locus; A_R: allelic richness; H_E and H_O: expected and observed heterozygosities, respectively; P_{HWE}: p value for Hardy–Weinberg Equilibrium; F_{IS}: inbreeding coefficient; P_{FIS}: p value for F_{IS}

Table 3 F'_{ST} and Jost's Dest (lower diagonal) and P values (upper diagonal) for geographical demes of *Astyanax caucanus* in the Cauca River

	S2/3	S4	S5	S6	S7/8
F'_{ST}					
S2/3	–	0.501	0.129	0.283	0.463
S4	0.013	–	0.288	0.063	0.227
S5	0.013	0.011	–	0.462	0.010
S6	0.012	0.014	0.009	–	0.333
S7/8	0.010	0.012	0.012	0.010	–
Jost's Dest					
S2/3	–	0.506	0.127	0.288	0.462
S4	− 0.001	–	0.285	0.063	0.223
S5	0.020	0.009	–	0.450	0.010
S6	0.010	0.030	0.000	–	0.321
S7/8	0.000	0.013	0.039	0.006	–

All P values were not significant after sequential Bonferroni correction

Fig. 3 Discriminant analysis of principal components (**A**) and coancestry plot suggested by Structure (**B**) of five demographic demes of *Astyanax caucanus* in the Cauca River

Stock2 showed high values of A_R. Additionally, *A. caucanus* exhibits significant values of F_{IS} coefficient > 0.380 indicating high inbreeding levels in the species. Furthermore, bottleneck analysis showed that this fish species experienced recent and severe reduction of population size given the values of M-ratio smaller than 0.680 in addition to significant values found in the I.A.M. and T.P.M. tests (Table 5). In addition, values of N_e were higher than 1000 in the two stocks (Stock1: 24,730, 95% CI: 22,878–26,439, autocorrelation value: 0.119; Stock2: 25,234, 95% CI: 23,417–26,978, autocorrelation value: 0.084). In the sample, 32 individuals were identified as likely migrants (P < 0.01) among river sections according to the results of the first-generation migrant detection test. Specifically, 17 individuals collected downstream of collection sectors were detected as likely immigrants from upstream sections (Stock1:
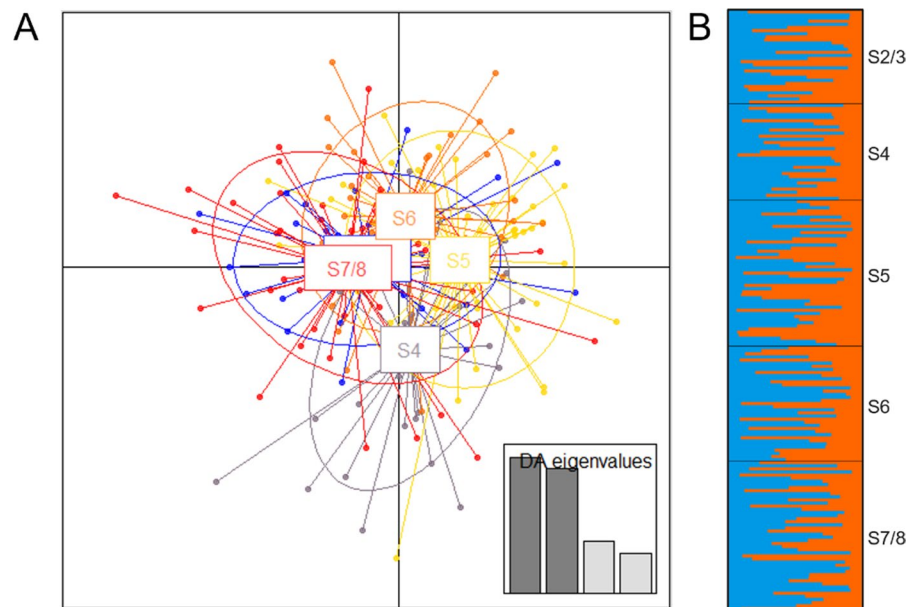
Table 4 Genetic diversity of *Astyanax caucanus* in all the sampling area (Cauca) and the two genetic stocks (Stock1 and Stock2) found in this study, using 12 microsatellite *loci*

Group	N	Na	A_R	H_O	H_E	P_{HWE}	F_{IS}	P_{FIS}
Cauca	193	16.750	16.734	0.436	0.840	**0.000**	0.407	**0.000**
Stock1	96	14.583	14.456	0.444	0.832	**0.000**	0.389	**0.000**
Stock2	97	14.000	13.914	0.427	0.827	**0.000**	0.408	**0.000**

Values in bold denote statistical significance (alpha = 0.050). N: sample size; Na: average number of alleles per locus; A_R: allelic richness; H_E and H_O: expected and observed heterozygosities, respectively; P_{HWE}: p value for Hardy–Weinberg Equilibrium; F_{IS}: inbreeding coefficient; P_{FIS}: p value for F_{IS}

Table 5 Tests to detect recent bottleneck in population of *Astyanax caucanus* from the Cauca River

Deme	N	I.A.M	S.M.M	T.P.M	M-ratio
S2/3	30	**< 0.001**	0.076	**< 0.001**	**0.225**
S4	31	**< 0.001**	0.575	**0.005**	**0.196**
S5	47	**< 0.001**	0.102	**< 0.001**	**0.211**
S6	37	**< 0.001**	0.715	**< 0.001**	**0.211**
S7/8	48	**< 0.001**	0.689	**0.002**	**0.212**
Stock1	96	**< 0.001**	0.830	**< 0.001**	**0.214**
Stock2	97	**< 0.001**	0.661	**< 0.001**	**0.213**
Cauca	193	**< 0.001**	0.765	**< 0.001**	**0.224**

Values in bold denote statistical significance (alpha = 0.05).

N sample size, *I.A.M.* infinite alleles model, *S.M.M.* stepwise mutation model, *T.P.M.* two phase mutation model

8; Stock2:9), while 15 individuals collected upstream was identified as likely immigrant from downstream sections (Stock1: 11; Stock2: 4).

Discussion

This study assessed the genetic status and structure of the short-distance migratory species *Astyanax caucanus* in the middle and lower sections of the Cauca River in Colombia. Due to the morphological similarities with other *Astyanax* species distributed in the studied area, species identification was supported by a phylogenetic analysis based on partial sequences of MT-COI that revealed three main groups. The haplogroup clustered with *A. caucanus* from Tarazá, hence, was considered as *A. caucanus* due to the inclusion of sequences from specimens unequivocally assigned to this species, coming from its type locality, also having nomenclatural priority over remaining species names applied to analyzed samples from the Magdalena-Cauca River hydrographic region. The two haplogroups that contain samples morphologically identified as *A.* aff. *fasciatus* are phylogenetically distant from *Psalidodon fasciatus* from Brazil supporting the restricted distribution recently proposed for this last species (Terán et al., 2020). Since some limitations of MT-COI have been reported for discrimination of some morphologically distinguishable species or with high intraspecific variation (Rosini et al., 2016; Terán et al., 2020), our outcomes indicate that an integrative revision is required to clarify the taxonomy of this genus in Colombia.

Although there are microsatellite *loci* developed for other *Astyanax* members (Strecker, 2003; Zaganini et al., 2012), this study developed a set of species-specific microsatellite *loci* for *Astyanax caucanus* aiming avoid problems associated to the use of heterologous microsatellite *loci* and the type of repeat motifs (see review: Guichoux et al., 2011). The newly developed set of 16 *loci* is characterized by high polymorphism level, good band resolution, specificity, desired fragment size, and high levels of expected heterozygosity. In addition, the *loci* show departures of Hardy–Weinberg equilibrium and deficit of observed heterozygosity in the analyzed sample, which may be explained by the high number of alleles per *locus* that demand a great sample size for analysis (see review: Abdul-Muneer, 2014), and biological causes that are discussed below such as Wahlund effect, inbreeding, and assortative mating.

Results reveal that geographic demes are not genetically differentiated supporting the hypothesis that *A. caucanus* exhibits gene flow along the analyzed area (325 km). This outcome is congruent with the detection of 32 individuals identified as likely migrants among river sections, as indicated by the results of the Bayesian analysis with Geneclass2 (Piry et al., 2004) that suggest the potential movement of individuals in both upstream and downstream directions, as well as the drift of larvae from upstream sections. This result differs from the pattern found in other congeners that show populations geographically structured (Strecker et al., 2003; Leuzzi et al., 2004; Peres et al., 2005; Hausdorf et al., 2011; Bradic et al., 2012; Freitas-Lidani et al., 2018; Limeira et al., 2022) even when they were separated by only 5 km (Sofia et al., 2006). If larval duration in *A. caucanus* reaches five days post fertilization as observed in related congeners (Axelrod et al., 1971; Hinaux et al., 2011; Stevanato & Ostrensky, 2018; dos Santos et al., 2020), *A. caucanus* larvae may travel distances even longer (up to 340 km) than the adults in the migration events (50 km; Jiménez-Segura et al., 2021), favoring gene flow. Among marine species, this explanation is usual since the gene flow is favored via larval dispersal (Hellberg, 1996; Matschiner et al., 2009; Pascual et al., 2017).

Despite the absence of spatial structure, *A. caucanus* exhibits two stocks that coexist in the middle and lower sections of the Cauca River. Due to the limitation of MT-COI and morphology for *Astyanax*

species discrimination (Rosini et al., 2016; Terán et al., 2020), the presence of two genetic stocks may be explained by morphological differences recognized in the names *A. caucanus* and *A. magdalenae*. However, there are other possible aspects in the life history of *A. caucanus* that could explain the presence of both stocks. One explanation considers the two annual spawning periods of this species (Jiménez-Segura et al., 2016), related to the bimodal hydrological cycle of the basin (López-Casas et al., 2016), which may reflect temporal reproductive isolation between the two genetic groups (Wahlund effect). Although the presence of sexually mature individuals from both stocks at the same hydrological period may not provide support to this explanation, other aspects such as assortative mating by preference of environmental factors (*e.g.,* temperature, light intensity, and/or timing), asynchronous spawning, or the temporal presence of hooks in the anal fin as was shown in *Psalidodon scabripinnis* (Jenyns, 1842) (Castro et al., 2014), might explain the reproductive isolation of both stocks. These hypotheses must be tested in further studies with more information regarding the reproductive characteristics of *A. caucanus*.

The coexistence of different genetic groups has been recorded for the genus, as it was also found in *Astyanax altiparanae* Garutti & Britski, 2000 in several sections of the Penacho stream (Ferreira et al., 2016) and in sympatric *Astyanax* morphs from Central America (Garita-Alvarado et al., 2021), although its causes remain to be clearly established. This genetic structure has been also found in other species that inhabit the Cauca River, such as *Prochilodus magdalenae* Steindachner, 1879 (Landínez-García et al., 2020), *Megaleporinus muyscorum* (Steindachner, 1900) (Márquez et al., 2021), *Pimelodus grosskopfii* Steindachner, 1879 (Restrepo-Escobar et al., 2021), and *Pimelodus yuma* Villa-Navarro & Acero P., 2017 (Joya et al., 2021). The coexistence of genetic groups in the Cauca River is explained by spatial or temporal reproductive isolation; nevertheless, both explanations require further research to clarify the probable causes of these patterns of genetic structuring (Márquez et al., 2021).

The two stocks in this study show high genetic diversity levels, specifically the average number of alleles/*locus* and expected heterozygosity are higher than the average values for Neotropical Characiformes (Na: 10.920; H_E: 0.675; Hilsdorf

& Hallerman, 2017). Particularly for *A. caucanus*, values of H_E (0.827–0.840) are higher than those found in *Astyanax* sp. (0.550; Strecker et al., 2012), *A. mexicanus* (De Philippi, 1853) (0.487; Strecker et al., 2003) and *P. scabripinnis* (0.487; Limeira et al., 2019), and similar to those found in *A. altiparanae* (0.833; Ferreira et al., 2016), and *A. bransfordii (Gill, 1877)*, *A. nicaraguensis* Eigenmann & Ogle, 1907 and *A. nasutus* Meek, 1907 (0.840; Garita-Alvarado et al., 2021). Compared to species of the Cauca River, *A. caucanus* shows higher values of H_E than those found in *B. henni* (0.604–0.662; Landínez-García & Márquez, 2020), *Ichthyoelephas longirostris* (Steindachner, 1879) (0.771–0.798; Landínez-García & Márquez, 2016), *Curimata mivartii* Steindachner, 1878 (0.793–0.810; Landínez-García & Marquez, 2018), *Sorubim cuspicaudus* Littmann, Burr & Nass, 2000 (0.771–0.785; Restrepo-Escobar et al., 2021), and *Pseudoplatystoma magdaleniatum* Buitrago-Suárez & Burr, 2007 (0.770–0.798; García-Castro et al., 2021), similar to those found in *Ageneiosus pardalis* Lütken, 1874 (0.832–0.839; Restrepo-Escobar et al., 2021), *Pimelodus yuma* (0.825–0.875; Joya et al., 2021), *Pseudopimelodus atricaudus* Restrepo-Gómez, Rangel-Medrano, Márquez & Ortega-Lara, 2020 (0.804–0.840; Rangel-Medrano & Márquez, 2021), and lower than those found in *Prochilodus magdalenae* (0.898; Landínez-García et al., 2020) and *Pimelodus grosskopfii* (0.849–0.866; Restrepo-Escobar et al., 2021).

The high levels of inbreeding of *A. caucanus* exceed by more than three times the threshold to avoid short-term decreases in reproductive performance in captive populations: 0.100 (Soulé, 1980). Although this is a threshold for captive populations, Frankham et al. (2014) indicate that any sign of inbreeding reduces population viability in wild populations. Due to the scarce information about the life cycle of *A. caucanus*, causes for such values of inbreeding remain unclear; however, Keller & Waller (2002) indicate that inbreeding occurs in wild populations regularly and can be severe but do not exclude the increase of environmentally inflicted mortality. Specifically, the elevated levels of inbreeding may stem from a nonrandom contribution by parents, possibly influenced by variations in their fertility, the viability of their offspring, or differences in their reproductive behavior (see: Caballero, 1994).

Alternatively, the heightened levels of inbreeding may be attributed to a recent and severe reduction in population size as estimated by Bottleneck analysis, a common characteristic showed by other species present in the Cauca River even before the construction of Ituango Hydropower (Landínez-García & Marquez, 2018; Landínez-García et al., 2020; García-Castro et al., 2021; Restrepo-Escobar et al., 2021). Since *A. caucanus* is not a fishery resource, anthropic activities different to the fishery and likely climate events may explain these results. Among the remaining anthropic harmful activities are mining, oil refinery, hydropower generation, urban water supply and dumping, agricultural crops, and livestock production (Angarita et al., 2021; Gutiérrez-Moreno & de la Parra-Guerra, 2021). They lead to chemical dumping (*e.g.,* heavy metals, pesticides, drugs), oil spill (accidental or terrorism related), sedimentation (30,000–300,000 m^3/day), hydrological disconnection, deforestation, among others (Angarita et al., 2021; Gutiérrez-Moreno & de la Parra-Guerra, 2021; Hernández-Barrero et al., 2021; Lasso et al., 2021).

It is important to recall that *A. caucanus* along with other congeners represent the dominant biomass of the river (Jiménez-Segura et al., 2021; Valderrama-Barco et al., 2021), hence, its population is still large, even after experiencing bottlenecks. This last idea is supported by the estimated value of the effective population size ($N_e > 1000$) that indicates little or no genetic threats to its evolutionary potential (Frankham et al., 2014). Nevertheless, consequently with the signals of genetic bottleneck and levels of inbreeding above discussed, careful conservation strategies focused on habitat preservation are required to lower risk of future reductions in population size.

In summary, this work found two genetic stocks that coexist, comigrate and exhibit gene flow along the middle and lower sections of the Cauca River. Each stock show high levels of expected heterozygosity and effective population numbers, in addition to evidence of recent and severe reductions in population size and high values of inbreeding coefficients. Likewise, this study developed a set of species-specific microsatellite *loci* that may help monitor the genetic diversity, structure, and demography of *A. caucanus*, information relevant for designing short-term regulations that are crucial to preserve the natural habitat of this tetra fish and support the food chain for commercially important species. Finally, this work provides insights on the genetic status of a species that is not influenced by fishing pressure.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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