

Different stocks of *Prochilodus lineatus* along the Cinzas River basin: contributions from a free-flowing tributary in the Capivara Reservoir region, Paranapanema River basin (upper Paraná River)



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Dams are one of the main threats to Neotropical potamodromous fish, causing blockages of migratory routes and loss of breeding areas in an increasing number of drainage systems. In this scenario, current data argue that the maintenance of free-flowing tributaries along fragmented watersheds would be an important strategy to the migratory fish conservation, both in panmixia situations and in scenarios with different coexisting stocks, as already reported for *Prochilodus lineatus*. This study analyzed microsatellite loci and mitochondrial haplotypes in samples of *P. lineatus* obtained in different years along the Cinzas River basin, as well as in the Capivara Reservoir (largest Reservoir in the Paranapanema main channel, upper Paraná River) and in the Tibagi River, all connected by free stretches, aiming to investigate the contributions of the Cinzas River basin to the population dynamics of migratory fish in the Capivara reservoir region. Both markers detected high genetic diversity levels and indicated different genetic stocks of *P. lineatus* along the Cinzas River basin, suggesting some temporal genetic structuring, as well as the mixture of these stocks in the sample from the Capivara reservoir and from other points, corroborating the relationships among stocks in this reservoir and its tributaries.

Keywords: Curimba, D-Loop, Microsatellite, Population genetics, Potamodromous fish.

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Barragens são umas das principais ameaças para peixes potamódromos Neotropicais, causando bloqueios de rotas migratórias e perdas de áreas de reprodução em um número crescente de sistemas de drenagem. Neste cenário, dados atuais defendem que a manutenção de tributários de fluxo livre em bacias hidrográficas fragmentadas seria uma importante estratégia na conservação de peixes migradores, tanto em situações de panmixia quanto em cenários com diferentes estoques coexistindo, tal como já relatado para *Prochilodus lineatus*. Este estudo analisou locos microssatélites e haplótipos mitocondriais em amostras de *P. lineatus* obtidas em diferentes anos ao longo da bacia do rio das Cinzas, no reservatório de Capivara (maior reservatório na calha principal do rio Paranapanema, alto rio Paraná) e no rio Tibagi, todos conectados por trechos livres, visando investigar as contribuições da bacia do rio das Cinzas na dinâmica populacional de peixes migradores na região do reservatório de Capivara. Ambos os marcadores detectaram altos níveis de diversidade genética e indicaram diferentes estoques de *P. lineatus* ao longo da bacia do rio das Cinzas, sugerindo alguma estruturação genética temporal, bem como a mistura destes estoques na amostra do reservatório de Capivara e de outros pontos, corroborando as relações de estoques neste reservatório e seus tributários.

Palavras-chave: Curimba, D-Loop, Genética de populações, Microssatélites, Peixe potamódromo.

INTRODUCTION

Breeding migrations of Neotropical potamodromous fishes are commonly reported as upstream migrations during the rainy season (period of higher hydrological connectivity), aiming to spawn in upper reaches and tributaries, which is followed by drift of eggs and larvae and development of young in wetlands and floodplains (Carolsfeld *et al.*, 2003). However, different migratory dynamics, ecological requirements and life history traits have been reported among these species (Carolsfeld *et al.*, 2003; Makrakis *et al.*, 2012; Pachla *et al.*, 2022), contributing to different patterns of population biology, such as panmictic populations over large geographic scales (Santos *et al.*, 2007; Carvalho-Costa *et al.*, 2008; Coimbra *et al.*, 2020), coexistence of genetic stocks in a single drainage (Hatanaka *et al.*, 2006; Sanches, Galetti Jr., 2007, 2012; Barroca *et al.*, 2012a), genetic structuring due to homing behaviors (Batista, Alves-Gomes, 2006; Pereira *et al.*, 2009), temporal population structuring (Rueda *et al.*, 2013; Braga-Silva, Galetti, 2016; Rosa *et al.*, 2022), as well as population differences due to anthropic interference (insertion of dams) (Garcez *et al.*, 2011; Esgúicero, Arcifa, 2010; Barroca *et al.*, 2012b).

Population genetic data from migratory fish are essential for understanding evolutionary and biological aspects, as well as for determining the appropriate spatial scale and required adaptations for the management and conservation actions (Carvalho, 1993; McIntyre *et al.*, 2016). In this context, there is a consensus that fluvial connectivity is a determining factor for the reproduction and recruitment of potamodromous species (Lucas *et al.*, 2001; Carolsfeld *et al.*, 2003; Pachla *et al.*, 2022), as well as for the gene

flow levels and the distribution range of populations (Sivasundar *et al.*, 2001; Santos *et al.*, 2007; Allendorf *et al.*, 2012). However, this connectivity has been increasingly compromised in hydrographic systems, mainly due to the insertion of hydroelectric dams, which block the migratory routes and eliminate lotic stretches required for the spawning, bringing great concerns with the decline of migratory fish populations (Agostinho *et al.*, 2016; Mastrochirico Filho *et al.*, 2018; Deinet *et al.*, 2020). In the case of Neotropical migratory fish, attempts to mitigate the dam impacts have mainly been the use of transposition mechanisms and river restocking (Makrakis *et al.*, 2007; Agostinho *et al.*, 2010), although the efficiency and applicability of these strategies have not yet been a consensus among researchers (Agostinho *et al.*, 2010; Kemp, O’Hanley, 2010; Pelicice *et al.*, 2015). On the other hand, recent studies, including population genetic studies (Ferreira *et al.*, 2017, 2022), fish eggs and larvae analysis (da Silva *et al.*, 2019; Azevedo-Santos *et al.*, 2021; Sulzbacher *et al.*, 2023), mark and recapture studies (Antonio *et al.*, 2007; Makrakis *et al.*, 2012) and ecological analysis (Marques *et al.*, 2018), increasingly highlight the preservation of free-flowing tributary rivers as an important strategy for the maintenance of fish diversity in dammed systems, including migratory fish populations and their genetic diversity levels.

In Paraguay-Paraná-Plata system, at least 20 species are migratory fish (Agostinho *et al.*, 1995; Carolsfeld *et al.*, 2003), including the curimba *Prochilodus lineatus* (Valenciennes, 1837), a detritivorous fish (medium to large body) that can drive extensive migrations (over 1,500 km) (Carolsfeld *et al.*, 2003). However, most of its distribution area, including the upper Paraná River basin (UPRB), is already highly fragmented by dams in the main channel and large tributaries (Makrakis *et al.*, 2019). Despite this scenario, some UPRB’s sub-basins such as the Paranapanema River (eleven HPPs in its main channel) still include tributaries (*e.g.*, Tibagi and Cinzas rivers) showing long free-flowing stretches that could contribute to the regional preservation of fish diversity, including populations of migratory fish. The Cinzas River basin, in particular, has been considered a Priority Area for Biodiversity Conservation by the Brazilian Ministry of the Environment (MMA, 2016) and it is already suggested, based on genetic data (Lopes *et al.*, 2007; Ferreira *et al.*, 2022), egg and larval analyses (Vianna, Nogueira, 2008; Frantini-Silva *et al.*, 2015; Lima *et al.*, 2020) and ichthyofauna surveys (Galindo *et al.*, 2020), as an important drainage in maintaining the biodiversity of fish in the Capivara Reservoir region (lower Paranapanema River).

The present study analyzed the population genetics of *P. lineatus* along the Cinzas River basin, including samples from large schools obtained in different breeding seasons (2011 and 2012) by Galindo *et al.* (2020), aiming to investigate the contribution of this tributary to the population dynamics of migratory fish in the Capivara Reservoir region. In this context, the study sought to test the hypotheses: *i*- schools in Cinzas River basin would include individuals migrating from the Capivara Reservoir; *ii*- distinct genetic stocks would be coexisting in the Cinzas River basin over time; *iii*- mixing of distinct genetic stocks could occur in the “transit area”, as well as due to influences from restocking programs and/or the fact that Cinzas River basin is acting as an alternative route for stocks that had their original route blocked in the Paranapanema River.

MATERIAL AND METHODS

Study area. The Cinzas River basin drains 9,658.8 km² within Paraná State, Brazil (Britto *et al.*, 2003; Vianna, Nogueira, 2008), constituting one of the main tributaries of the left bank of the Paranapanema River, upper Paraná River basin (Britto *et al.*, 2003) (Fig. 1). The source of Cinzas River is in Serra de Furnas (24°27'34"S 49°55'49"W) and its main channel extends for approximately 370 km (in a south–north direction) until its confluence with the Paranapanema River (23°01'03.51"S 50°24'22.68"W), in the Capivara Reservoir (about 576 km²). Its main tributary, the Laranjinha River, extends about 350 km and has only a small dam along the main channel, originating from a Small Hydropower Plant (SHP) which never went into operation (since its construction in the 1950s) and has a fish pass system since 2006 (Makrakis *et al.*, 2019; Galindo *et al.*, 2020). The Cinzas River also has only a small dam in its main channel, however, it is near headwaters and upstream Salto Cavalcante, a 20 m high fall in Tomazina municipality (Vianna, Nogueira, 2008).

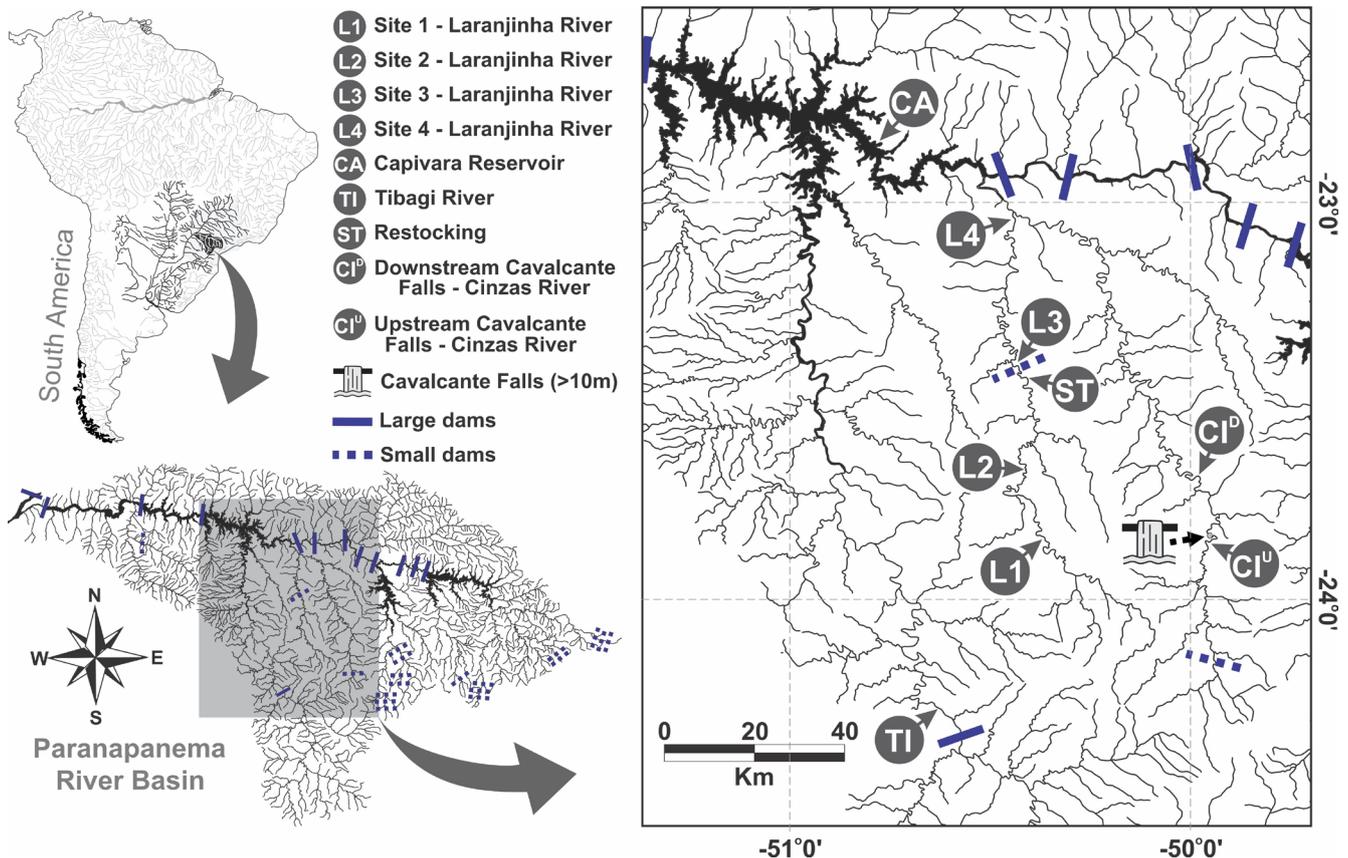


FIGURE 1 | Sampling locations for *Prochilodus lineatus* along the Capivara Reservoir region (Paranapanema River basin), including the Laranjinha River (L1–L4), Cinzas River main channel (CI^U - upstream and CI^D - downstream from Salto Cavalcante falls), Capivara Reservoir (CA) and Tibagi River (TI), as well as the site (ST) of a restocking event in the Laranjinha River. Source: modified from Google Earth, 2018 (<https://www.google.com.br/maps>).

Fish sampling. Almost all samples analyzed in the present study were collected in breeding seasons of different years, including only adult individuals (body lengths above 25 cm). Most were obtained by Galindo *et al.* (2020) along Laranjinha River main channel, including a sample (L4 = 12) obtained near the confluence with the Cinzas River (23°01'53.90"S 50°26'51.90"W), collected on December 28, 2011 (Dec_11), a sample (L3 = 24) of individuals using the fish pass system to cross the small dam in the main channel (23°17'49.95"S 50°28'43.27"W), collected on March 1st and 2nd, 2011 (Mar_11), and two samples from large schools (reproductively mature) recorded in two points (L2 = 24 and L1 = 34) along the upper main channel section at the end of breeding season. L2 was sampled in Ibaiti Municipality (23°43'31.88"S 50°26'34.42"W) on March 4, 2012 (Mar_12a), and L1 was sampled about 40 km upstream of L2, in Figueira Municipality (23°51'34.73"S 50°22'43.04"W) on March 11, 2012 (Mar_12b). In addition, the study also analyzed a sample of fingerlings (ST = 30, origin not informed because their data require confidential treatment) released into the Laranjinha River (23°25'28.65"S 50°26'43.82"W) on September 2009 (Sep_09), seeking to investigate the care taken with restocking along this basin in periods preceding the study of Galindo *et al.* (2020), as well as 15 individuals from Cinzas River main channel, 13 downstream (CI^D, 23°38'57.27"S 50°02'28.48"W) and two upstream (CI^U, 23°51'13.39"S 49°56'47.52"W) from Salto Cavalcante (obtained on October 10 and November 3, 2019, Oct-Nov_19), aiming to understand the migration limits for *P. lineatus* in the Cinzas River main channel. To investigate the origin of the stocks, all samples from the Cinzas River basin (Cinzas+Laranjinha) were compared with a sample from the Capivara Reservoir (CA = 30), collected on March 27 and April 4, 2010 (Mar-Apr_10), and a sample from the Tibagi River (TI = 13), obtained in February 2012 (Feb_12).

Samples were obtained from 48 h samplings using gill nets, cast nets and fishing rods. Tissue samples were placed in 70% alcohol and stored at -20°C. Voucher specimens were deposited in the Museu de Zoologia of the Universidade Estadual de Londrina, Londrina (accession number: MZUEL 9437).

Microsatellite loci and mitochondrial haplotypes (D-loop). Total genomic DNA was isolated from rayed fins or muscle tissue using a phenol-chloroform protocol and subsequently quantified and diluted following the steps in Ferreira *et al.* (2017). A total of seven microsatellite loci, including PL03, PL34, PL119, PL139 (Rueda *et al.*, 2011) and Pli34, Pli43 and Pli60 (Yazbeck, Kalapothakis, 2007), were analyzed on 182 *P. lineatus* individuals. Polymerase Chain Reactions (PCR) were conducted using concentrations and conditions employed by Ferreira *et al.* (2017), including specific primer annealing temperatures from Yazbeck, Kalapothakis (2007) and Rueda *et al.* (2011), as well as the method from Schuelke (2000) for labeling PCR products with fluorescence (FAM, HEX, NED and PET). Genotyping was performed in an ABI PRISM 3500-XL automated sequencer (Applied Biosystems) using GeneScan 600 Liz (Applied Biosystems) as a molecular weight marker and the fragment length analysis was conducted using GeneMarker 1.85 software (Soft Genetics, State College, PA, USA).

In the Mitochondrial DNA (mtDNA) analysis, part of the D-Loop region was amplified for all samples using the primers L 5'-AGAGCGTCGGTCTTGTAACC-3' (Cronin *et al.*, 1993) and H 5'-CCTGAAGTAGGAACCAGATG-3' (Meyer *et al.*, 1990), as well as the PCR conditions, reagent concentration and purification step from Ferreira *et al.*

(2017). Both strands (H and L) were sequenced using the Big Dye Terminator v. 3.1 kit (Applied Biosystems), according to the manufacturer's instructions. The readings were carried out on an ABI-PRISM3500 XL automated sequencer and the sequences were edited and aligned using MEGA 5.0 (Tamura *et al.*, 2011). The different haplotypes found were deposited in GenBank (OR188144 – OR188190). The species identities of amplified fragments were confirmed by BLAST searches - Basic Local Alignment Search Tool (Altschul *et al.*, 1990).

Molecular data analysis. Microsatellite data were first analyzed using the Micro-Checker v. 2.2.1 program (Van Oosterhout *et al.*, 2004) to analyze missing data and to evaluate the presence of null alleles and scoring errors due to allelic dropout and stutter peaks. Deviations from the Hardy-Weinberg Equilibrium (HWE) and the occurrence of linkage disequilibrium (LD) were tested in the Genepop v. 1.2 program (Raymond, Rousset, 1995), with 1,000 iterations and 1,000 dememorization, and the sequential Bonferroni correction (Rice, 1989) was used to adjust the alpha values. Popgen v. 1.31 program (Yeh *et al.*, 1999) was used to estimate the number of alleles (A), expected heterozygosity (H_E), observed heterozygosity (H_O), mean alleles per locus (N_A), number of effective alleles (N_E) and number of private alleles (N_p). Significant inbreeding (F_{IS}) values ($P < 0.05$) and the allelic richness (R_A), using a rarefaction approach (corrected for a minimum sample size of ten diploid individuals), were estimated using Fstat v. 2.9.3 program (Goudet, 2001).

On mtDNA data, the number of haplotypes (Nh), haplotype diversity (h) and nucleotide diversity (π) were obtained using the DnaSP v. 5 program (Librado, Rozas, 2009), while Network 4.6.1.1 (Fluxus Technology Ltd - <http://www.fluxus-engineering.com>) was used to construct the haplotype network based on the median-joining algorithm (Bandelt *et al.*, 1999). Aiming to investigate the care taken with restocking programs along Capivara Reservoir region, including possible negligence in the release of fingerlings produced from matrices originating from watersheds with genetic stocks different from those naturally present in the study area, a total of 304 D-Loop sequences of *P. lineatus* obtained in previous studies and available in Genbank were analyzed (Tab. S1). From this, only those sequences identical to haplotypes from the present study were included in the haplotype network.

To investigate the demographic history, Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) statistics were estimated on mtDNA data using Arlequin v. 3.5.1.3 (Excoffier, Lischer, 2010) and the pairwise mismatch distributions were obtained with DnaSP v. 5 (Librado, Rozas, 2009). At the same time, Bottleneck v. 1.2.02 program (Piry *et al.*, 1999) was run on microsatellite data to investigate signs of recent population bottlenecks (indicated by deviations from the mutation-drift equilibrium) using the Wilcoxon signed rank test (Luikart, Cornuet, 1998) of heterozygosity excess under Infinite Alleles Model (IAM), Two-Phase Model (TPM - with 90% SMM and 10% IAM) and Stepwise Mutation Model (SMM), as well as the "Mode-shift test" that indicates bottlenecks resulting from alterations in allele frequency distributions (Luikart *et al.*, 1998).

For population differentiation analyses, the pairwise D_{EST} values (Jost's genetic differentiation estimator) (Jost, 2008) were obtained from microsatellite data in the R statistical environment (R Development Core Team, 2021) with the DEMETics package (Jueterbock *et al.*, 2012). From these data were also obtained the F_{ST} and R_{ST} values

in the Arlequin v. 3.5.1.3 (Excoffier, Lischer, 2010), using significant estimates based on 10,000 permutations. On mtDNA data, pairwise Φ_{ST} values (analogous to Wright's F-statistics) were calculated in Arlequin v. 3.5.1.3 (Excoffier, Lischer, 2010) using the Tamura model (Tamura, 1992), which was indicated as the best-fit nucleotide sequence evolution model for the data according to ModelTest v. 3.7 (Posada, Crandall, 1998).

A Bayesian cluster analysis was also performed on the microsatellite data in Structure v. 2.3.3 program (Pritchard *et al.*, 2000), running twenty replicates for each *K* value (number of clusters), from *K* = 1–11, including a burn-in of 10,000 Markov Chain Monte Carlo (MCMC) iterations, followed by 100,000 MCMC of data collection. Structure Harvester (Earl, VonHoldt, 2012) was used to estimate the optimal *K*, while Clumpp 1.1.2 (Jakobsson, Rosenberg, 2007) summarized the best *K* runs and Distruct 1.1 (Rosenberg, 2004) plotted the results on a graph.

RESULTS

Population structure. Micro-Checker program showed no presence of scoring error or allele dropout for microsatellite loci. Possible null alleles and homozygote excess were indicated only for PL139 (all samples), PL03 (L2, CI and TI), PL34 (L3, L4 and CA) and Pli60 (L2, L3, L4, ST and CA). However, independent runs, including and excluding these loci, showed similar results, thus all loci were considered for analysis. Among the genetic differentiation estimators applied on microsatellite data, almost all pairwise D_{EST} and F_{ST} values were significant, ranging from 0.029 (L3 x L4) to 0.599 (L3 x TI) and from 0.018 (L1 x L3) to 0.599 (L3 x TI), respectively. On the other hand, just half of the pairwise R_{ST} values were significant, ranging from 0.062 (L1 x L2) to 0.154 (ST x CI). Meanwhile, only eight pairwise Φ_{ST} values (estimated from mtDNA data) were significant, ranging from 0.054 (L1 x CA) to 0.189 (L3 x TI) (Tab. 1).

TABLE 1 | Pairwise genetic differentiation among samples of *Prochilodus lineatus* obtained along the Capivara Reservoir region (Paranapanema River basin), including the Laranjinha River (L1–L4), Cinzas River main channel (CI), Capivara Reservoir (CA), Tibagi River (TI) and a restocking sample (ST). Below diagonal – pairwise D_{EST} /pairwise R_{ST} from microsatellite data. Above diagonal – pairwise F_{ST} from microsatellite data/pairwise Φ_{ST} from mtDNA data. *Significant values – P value < 0.05.

	L1	L2	L3	L4	ST	CI	CA	TI
L1		0.018*/0.019	0.121*/0.089*	0.068*/0.068	0.098*/0.069*	0.024*/0.011	0.084*/0.054*	0.080*/0.129*
L2	0.083*/0.018		0.110*/0.083*	0.064*/0.039	0.084*/0.013	0.023*/0.018	0.081*/0.011	0.083*/0.021
L3	0.420*/0.062*	0.372*/0.068*		0.020 /0.012	0.149*/0.069	0.060*/0.031	0.021*/0.037*	0.175*/0.189*
L4	0.258*/0.018	0.231*/0.026	0.029*/0.007		0.129*/0.019	0.014 /0.006	0.025*/0.033	0.128*/0.113
ST	0.400*/0.086*	0.339*/0.108*	0.428*/0.115	0.403*/0.091		0.066*/0.023	0.114*/0.006	0.051*/0.010
CI	0.165*/0.031	0.140*/0.063*	0.202*/0.006	0.034 /0.015	0.259*/0.154*		0.024*/0.029	0.045*/0.124*
CA	0.349*/0.063*	0.340*/0.071*	0.038 /0.005	0.025 /0.001	0.394*/0.146*	0.058*/0.001		0.123*/0.041
TI	0.402*/0.044*	0.419*/0.066*	0.599*/0.066*	0.485*/0.031	0.165*/0.120*	0.268*/0.014	0.513*/0.068*	

In the Bayesian clustering analysis performed on microsatellite data, both the $\ln \text{Pr}(X/K)$ (Pritchard *et al.*, 2000) and ΔK ad hoc statistics (Evanno *et al.*, 2005) indicated $K = 3$ as the most probable K value (cluster number) (Figs. 2A–B). The graphic representation of this analysis shows almost all samples including a mixture of individuals from different clusters, although L1 showed a distribution closer to that observed in L2 and L3 was closer to the distribution in L4, CI and CA (Fig. 2C).

Still on microsatellite data, all samples showed significant and positive inbreeding coefficient values (F_{IS}) and, after sequential Bonferroni correction for multiple comparisons, five significant ($\alpha = 0.05$, $k = 21$) linkage disequilibrium (LD) values were detected at L1 (pairs: PL34 x Pli43, PL119 x Pli43, PL119 x Pli60 and Pli43 and Pli34), while TI showed two values (pairs: PL119 x Pli60 and PL139 x Pli43) and L3 (Pli43 x Pli60), L4 (Pli43 x Pli60) and CA (PL119 x PL119) showed only one significant value. The number of microsatellite loci showing significant deviations (sequential Bonferroni correction - $\alpha = 0.05$, $k = 7$) from Hardy-Weinberg proportions ranged from two (L2 - PL03 and PL139, ST - Pli34 and Pli60) to six (CA - Pli34, Pli60, PL03, PL34, PL119 and PL139) per sample (Tab. 2).

Genetic diversity. In general, a total of 180 different microsatellite alleles were obtained, ranging from 60 (L4) to 109 (CA) per sample. The H_O and H_E estimates ranged from 0.490 (L4) to 0.777 (L1) and from 0.667 (L3) to 0.816, respectively. The highest values of private alleles (16), mean of alleles per locus ($N_A = 15.571$) and effective alleles ($N_E = 8.607$) were found at CA, while the lowest values occurred at L3 ($N_p =$ one $N_E = 5.341$) and L4 ($N_A = 8.571$). The allelic richness (R_A), corrected for a minimum sample size of ten diploid individuals, ranged from 6.918 (ST) to 9.845 (CI) (Tab. 2).

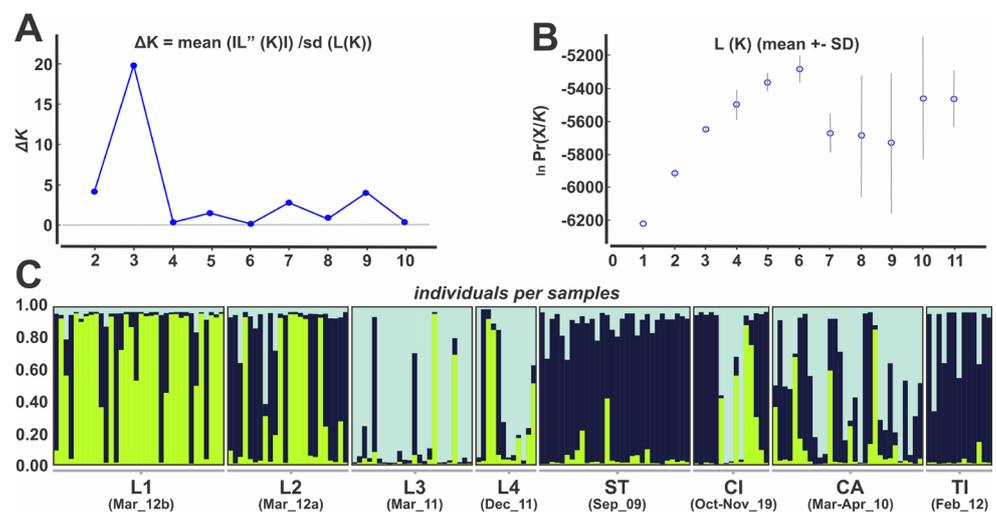


FIGURE 2 | Results of Bayesian clustering analysis (STRUCTURE) for *Prochilodus lineatus* samples obtained along in Laranjinha River (L1–L4), Cinzas River main channel (CI), Capivara Reservoir (CA), Tibagi River (TI) and a restocking event (ST). Estimates of the number of K groups based on mean likelihood $\ln(K)$ (A) and ΔK statistic (B). Graphical representation based on $K = 3$ (C). Each column represents a different individual and the colors represent the probability membership coefficient of that individual for each genetic cluster.

TABLE 2 | Genetic diversity of *Prochilodus lineatus* along Cinzas River basin, Capivara Reservoir and Tibagi River from microsatellite loci and mtDNA (D-loop) haplotypes. *N* = number of individuals analyzed, *A* = total number of alleles found per sample, *N_p* = number of private alleles, *R_A* = allelic richness, *N_A* = mean number of alleles, *N_E* = mean number of effective alleles, *H_o* = observed heterozygosity, *H_e* = expected heterozygosity, *F_{IS}* = rate of inbreeding, HWE = loci deviated from Hardy-Weinberg proportions, *Nh* = number of haplotypes found, *h* = haplotype diversity, π = nucleotide diversity, *D* = Tajima’s neutrality test (Tajima, 1989), *F_s* = Fu neutrality test (Fu, 1997). *Significant values – *P* value < 0.05.

Sample/ date	Microsatellite loci										mtDNA				
	<i>N</i>	<i>A</i>	<i>N_p</i>	<i>R_A</i>	<i>N_A</i>	<i>N_E</i>	<i>H_o</i>	<i>H_e</i>	<i>F_{IS}</i>	<i>EHW</i>	<i>Nh</i>	<i>h</i>	π	<i>D</i>	<i>F_s</i>
L1 (Mar_12b)	34	84	4	7.877	12.000	6.236	0.777	0.810	0.055*	4 ^{134, i43, 119, 139}	7	0.745	0.017	1.287	6.140
L2 (Mar_12a)	24	90	3	8.996	12.857	7.393	0.692	0.816	0.172*	2 ^{03, 139}	9	0.851	0.018	0.579	2.681
L3 (Mar_11)	24	71	1	6.978	10.143	5.341	0.497	0.667	0.274*	4 ^{134, i60, 34, 139}	7	0.688	0.014	0.296	3.303
L4 (Dec_11)	12	60	2	7.944	8.571	5.431	0.490	0.697	0.336*	4 ^{134, i60, 34, 139}	5	0.803	0.018	0.373	-0.631
ST (Sep_09)	30	71	4	6.918	10.143	5.428	0.603	0.740	0.201*	2 ^{134, i60}	12	0.862	0.014	0.357	1.076
CI (Oct-Nov_19)	15	85	5	9.845	12.143	7.602	0.624	0.803	0.256*	3 ^{134, 03, 139}	7	0.781	0.016	-0.196	4.301
CA (Mar-Apr_10)	30	109	16	9.010	15.571	8.607	0.600	0.753	0.220*	6 ^{134, i60, 03, 34, 119, 139}	19	0.961	0.019	-0.978	-3.853
TI (Feb_12)	13	64	5	8.150	9.143	6.449	0.608	0.773	0.251*	5 ^{134, i60, 03, 119, 139}	5	0.756	0.012	0.797	7.878
General	182	180			25.714	9.964	0.628	0.835	0.250*	7 ^{all loci}	47	0.861	0.016	0.314	2.612

A total of 57 polymorphic sites and 47 different haplotypes were obtained in the analysis of 516-bp (base pairs) from D-loop region of mitochondrial DNA. The number of haplotypes per sample ranged from five (L4 and TI) to 19 (CA). The highest values of number of haplotypes (*Nh* = 19), haplotype diversity (*h* = 0.961) and nucleotide diversity (π = 0.019) were found in the CA sample, while L3 (*h* = 0.688) and TI (*Nh* = 5, π = 0.012) (Tab. 2).

The haplotype network showed several mutational steps among the haplotypes found. H3 was the most frequent and 17 haplotypes (H23–H25, H27, H30–H33, H37–H39 and H42–H47) were singletons (Fig. 3). Among the 304 D-Loop sequences available in Genbank, 15 were identical to haplotypes from the present study (Fig. 3; Tab. S1), encompassing haplotypes previously obtained in Mogi-Guaçu River (H1, H2, H4, H5, H7, H10, H13 and H15) (Sivasundar *et al.*, 2001; Rosa *et al.*, 2022), Verde River (H6), Peixe River (H7), Iguaçu River (H14), Aguapeí River (H13), Pardo River (H1, H3, H4 and H5) and Paraná River main channel (H2, H8, H9, H10, H11 and H12) (Ferreira *et al.*, 2017).

Demographic analyses. In the neutrality tests (mtDNA data), all Tajima test (*D*) values and Fu test (*F_s*) values were not significant (Tab. 2). The mismatch distribution graphic showed a multimodal distribution for haplotypes in all samples (Fig. 3). In microsatellite data, the Wilcoxon sign-rank test detected heterozygosity excess (recent bottleneck signal) in only two samples (L1 and TI), but just under the IAM model, and all samples showed a typical L-shaped distribution (non-bottleneck) in the mode-shift test (Tab. S2).

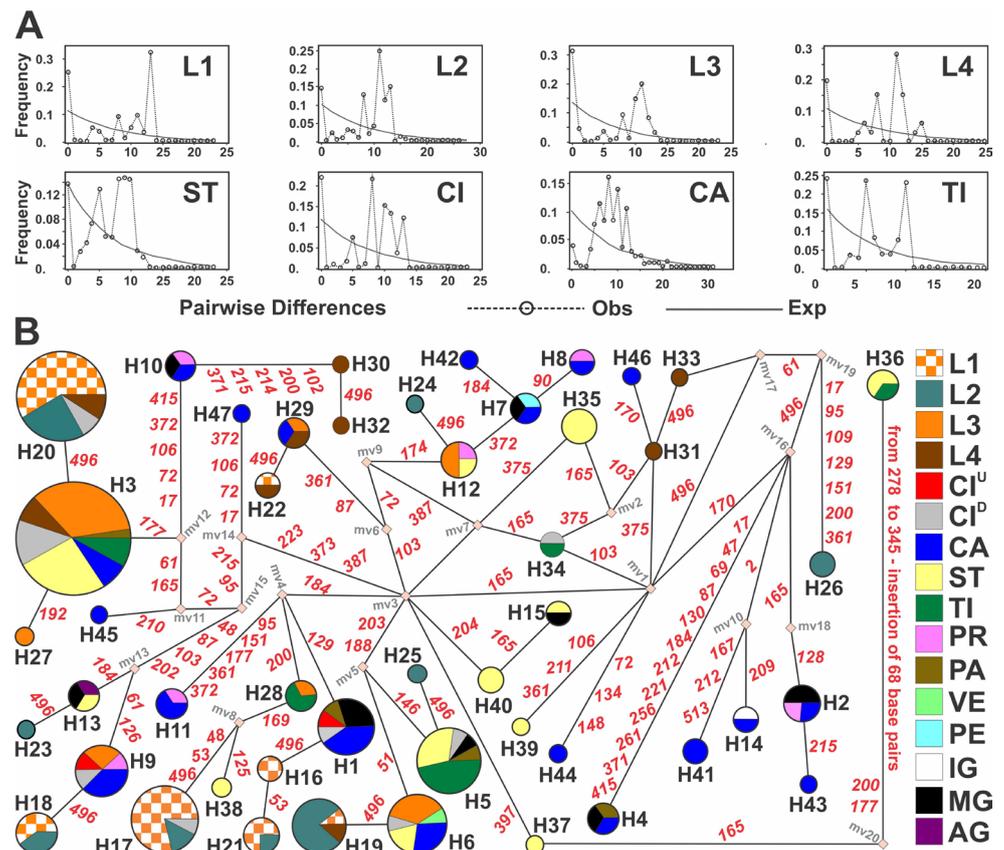


FIGURE 3 | Results from mtDNA (D-Loop) of *Prochilodus lineatus* in Cinzas River basin, Capivara Reservoir and Tibagi River. **A.** Mismatch distributions of mitochondrial haplotypes for all samples. **B.** Haplotype network, including GenBank data revealing previous records of some haplotypes in Paraná River main channel (PR) and in the Pardo (PA), Mogi-Guaçu (MG), Verde (VE), Peixe (PE), Aguapeí (AG), and Iguaçu (IG) rivers. Circle sizes are proportional to haplotype frequency.

DISCUSSION

Cinzas River basin's contributions in the Capivara Reservoir region. In general, the low or non-significant genetic structure (D_{EST} , R_{ST} , F_{ST} and Φ_{ST} estimators) that CA showed in relation to L3, L4 and CI corroborate the scenario where the Cinzas River basin would be providing habitats for spawning and recruitment of migratory fish in the Capivara Reservoir region, as already suggested by some fish egg and larval studies (Vianna, Nogueira, 2008; Frantine-Silva *et al.*, 2015; Lima *et al.*, 2020), ichthyofauna surveys (Hoffmann *et al.*, 2005; Galindo *et al.*, 2020), as well as a previous population genetic study of another migratory species (*Pimelodus maculatus* Lacepède, 1803) in the Laranjinha and Cinzas rivers (Ferreira *et al.*, 2022). Additionally, although L1 and L2 showed significant genetic structuring values in relation to L3, L4, CI and CA, all different genetic stocks detected among the samples from Cinzas River basin (discussed in the next topic), as well as that predominant in TI, seem to be represented within CA, as clearly evidenced by Bayesian cluster analysis and haplotype network, which also corroborates the contributions from the tributaries in the Capivara Reservoir region.

Capivara Reservoir is the largest reservoir in the Paranapanema River basin, covering an area of about 576 km² (Vianna, Nogueira, 2008). Considering that *Prochilodus* genus includes reports of different genetic stocks coexisting in single drainage (Hatanaka *et al.*, 2006; Barroca *et al.*, 2012a), as well as evidences of seasonal (Rueda *et al.*, 2013) and temporal (Rosa *et al.*, 2022) population structuring of *P. lineatus* in Paraguay-Paraná-Plata system, it seems plausible that different genetic stocks of this species would be occurring along the Capivara Reservoir and its tributaries. In fact, CA showed several results suggesting that it would have encompassed individuals from different genetic stocks, such as high numbers of microsatellite alleles and mitochondrial haplotypes, high h values (> 0.5) combined with high π values ($> 0.5\%$) and multimodal distribution in the mismatch distribution test (which reinforces the indication of the encounter of different lineages, according to Grant, Bowen, 1998 and Rogers, Harpending, 1992, respectively), the heterogeneous distribution in Bayesian cluster analysis, a significant F_{IS} value and the large number of microsatellites (six) deviating from the HW. These last two results, in particular, are some of the expected consequences from the inclusion of elements from multiple genetic units in a single sample, which creates an imbalance between the expected and observed heterozygosities (Freeland, 2005). This scenario seems plausible for CA, mainly because its sampling took place on different dates in 2010 (March 27 and April 4), as well as due to the fact that different genetic stocks, including those that reproduced in Paranapanema River main channel in a pre-damming period and those that use the Tibagi and Cinzas river basins, could have representatives coexisting throughout the large area of the Capivara Reservoir at some moment in the life cycle.

Different genetic stocks in the Cinzas River basin. Data from both genetic markers indicated different genetic stocks migrating along the Cinzas River basin, reinforcing the importance of this drainage for maintaining migratory fish populations. Interestingly, the predominant genetic stock in L3 was different from those predominant in L1 and L2, suggesting some temporal variation in the migration of schools composed of different genetic stocks. According to Bayesian cluster analysis (Fig. 2C), L1 and L2 would encompass two genetic stocks (green stock and dark blue stock) that would have reached reproductive areas in the upper reaches of the Laranjinha River in March 2012. Meanwhile, L3 encompassed another genetic stock (light blue stock), which was possibly migrating from downstream stretches and did not get past the small dam in the middle stretch of the Laranjinha River, so it used the fish ladder (where it was sampled in March 2011) on the side of the dam to overcome the obstacle and look for reproductive areas upstream. As already discussed above, this genetic stock could also be coming from the Capivara Reservoir, since CA (sampled in 2010) shows a group of “light blue” individuals in Structure graphical representation (which is corroborated by the haplotype sharing shown in the haplotype network). Additionally, although L1 and L2 (March, 2012) were sampled one year after the sampling of L3 (March, 2011), it seems plausible that both different genetic stocks (green, light blue and dark blue) would be migrating along the Laranjinha River in both 2011 and 2012 breeding seasons, especially when considering the possible occurrence of temporal genetic structuring and the success of different spawning waves among stocks.

Previous studies have reported temporal population structuring for *P. lineatus* (Rueda *et al.*, 2013; Perini *et al.*, 2021; Rosa *et al.*, 2022), as well as for other potamodromous in the Paraguay-Paraná-Plata system (Sanches, Galetti Jr., 2007; Ribolli *et al.*, 2017) and in other Neotropical drainage systems (Berdugo, Barandica, 2014; Braga-Silva, Galetti Jr., 2016; Lopes *et al.*, 2019). These studies point out, among other aspects, the possible influence of genetically-distinct spawning waves (constituted by different stocks) that breed in the same place at different time periods with some overlap (Jørgensen *et al.*, 2005). Variations in the hydrological cycles of a hydrographic system throughout the breeding season, for example, seem to be among the factors that contribute to some spawning waves occurring earlier and others later (Berdugo, Barandica, 2014; Braga-Silva, Galetti Jr., 2016; Ribolli *et al.*, 2017). Although the present study did not compare samples from different breeding seasons obtained at the same site, different spawning waves seem acceptable for the scenario already discussed above for L1, L2 and L3 in the Laranjinha River.

Mixing of distinct genetic stocks. All samples in the present study, including TI and ST, seem to be influenced by some level (greater or lesser) of mixing of distinct genetic stocks. However, relationships with stock dynamics were more coherent from L1, L2 and L3, which were sampled on a single date and showed sample size $> N = 20$. In case of L1 and L2, since these samples were obtained over a short time scale (one week) and a short spatial distance (about 40 km), the low or non-significant difference between them suggests that the same genetic stocks were sampled in different locations during its upstream movement. At the same time, the detection of different genetic stocks in these samples, as well as in L3 (on a smaller level), raise several questions about the factors influencing population dynamics. Since L1, L2 and L3 encompassed only adult individuals (body lengths above 25 cm), some gene flow among different stocks during reproduction seems plausible, especially in a possible situation of overlap of distinct spawning waves (Jørgensen *et al.*, 2005). Additionally, it is also important to consider that adults return to downstream stretches (areas of greater resource availability) after spawning (Carolsfeld *et al.*, 2003) and this could also favor the sampling of individuals from different genetic stocks in a single site.

In the case of CA, although this sample was obtained on different dates, its data suggests sampling in a “transit area” of different genetic stocks in Paranapanema River main channel, as already suggested for *P. lineatus* in a stretch of the Uruguay River (Rueda *et al.*, 2013). At the same time, this discussion is more difficult for L4, since its sample size was low ($N = 12$), as well as for CI, which had a low sample size ($N = 15$) and was obtained eight years after the other sites. Despite this, CI data contributed indicating that individuals obtained from upstream Salto Cavalcante falls belong to those stocks sampled downstream, suggesting that individuals of *P. lineatus*, eventually, can overcome this obstacle, possibly in periods of high floods.

Influences from restocking. In general, the sample from a restocking (ST) showed high genetic diversity levels, including 12 different mtDNA haplotypes (second largest in the analysis), indicating that captive breeders included a large number of females. At the same time, although Bayesian cluster analysis and some haplotypes, such as H36 (found only in TI and ST), indicated a greater relationship between TI and ST, the

broodstocks that resulted in ST also seems to include individuals from Cinzas River basin, suggesting that the release of fingerlings from a same farmed fish stock in Tibagi and Cinzas River basins could also have contributed to some mixing of stocks. It is noteworthy that the H36 has an insertion of 68 base pairs, however the research in BLAST and the analysis of cytochrome oxidase subunit I gene (GenBank: OR269255 – OR26925, results not shown) indicated that H36 belongs to *P. lineatus*.

The elaboration of restocking strategies admittedly requires data on the structure and genetic diversity of wild stocks (recipient populations), as well as captive broodstocks (Padhi, 2013; Casimiro *et al.*, 2022), aiming to minimize various damages, including the compromises of the local adaptation patterns, inbreeding depression events, genetic variability losses and changes in the genetic integrity of the wild stocks (Lorenzen *et al.*, 2012; Valiquette *et al.*, 2014; Oliveira-Farias *et al.*, 2022). Although restocking actions using *P. lineatus* have been reported along the Paranapanema River basin (Lopera-Barrero *et al.*, 2008; Casimiro *et al.*, 2022), the origin of ST was not informed because their data require confidential treatment. In any case, mtDNA data from this sample also draw attention to the care that possibly was taken in obtaining broodstocks, seeking to include only individuals from the Capivara Reservoir region. In fact, all ST haplotypes were the same or very close to those obtained in the other samples of the present study. Furthermore, even those haplotypes that coincided with haplotypes from the database (H12 – Paraná River main channel, H3 and H5 – Pardo River, H5, H13 and H15 – Mogi-Guaçu River, H6 – Verde River) also occurred in samples from the Capivara Reservoir and its tributaries, suggesting that these haplotypes would be in ST due to their wide distribution in the upper Paraná River basin and not due to the search for matrices outside the Paranapanema River basin.

Influences from the use of alternative routes. Considering a pre-fragmentation moment in the study area, stocks migrating along upstream stretches in the Paranapanema River main channel could be different from those using the Tibagi and Cinzas river basins. Thus, after the fragmentation of the Paranapanema River (including a dam a few meters upstream of Cinzas River mouth), stocks that previously migrated along the main channel (now blocked by dams) could look for an alternative for their migration and recruitment in unimpeded stretches of tributaries, such as in the Cinzas and Tibagi river basins, also contributing to some mixing of stocks, as detected in the present study.

The use of alternative migration routes in post-fragmentation scenarios has already been discussed for Neotropical potamodromous (Makrakis *et al.*, 2012; Baggio *et al.*, 2018; Casarim *et al.*, 2018; Ferreira *et al.*, 2022), including *P. lineatus* (Antonio *et al.*, 2007; Ferreira *et al.*, 2017), in different dam-fragmented watersheds. In the case of potamodromous species showing some level of homing behavior, Baggio *et al.* (2018) argue that the post-fragmentation use of alternative migration routes could lead to genetic homogenization of subpopulations in the short-term, since this scenario could favor interbreeding among different genetic stocks that used different routes. However, the influences herein suggested for the use of alternative routes seem more related to the heterogeneity of samples and not to genetic homogenization scenarios, mainly because several significant values indicating both temporal and spatial genetic structuring were obtained among samples, within and between drainages. Homing behaviors, that is, the return to natal nursery regions for reproduction, have already been suggested within

the *Prochilodus* genus (Godinho, Kynard, 2006) and are recognized for leading to a spatial population structure between drainages of a watershed, as reported for large Neotropical migratory catfish (Batista, Alves-Gomes, 2006; Pereira *et al.*, 2009). However, the heterogeneity detected in the Cinzas River basin, including individuals from different genetic stocks (some well related to TI), as well as the analysis of only one sample from Tibagi River, make it difficult to discuss the influences of homing behaviors on genetic differences among TI and those samples in Cinzas River basin.

In conclusion, present study identified different genetic stocks of *P. lineatus* migrating along the Cinzas River basin, possibly including some temporal genetic structuring, mix of different stocks in some areas, as well as the occurrence of these same stocks in the Paranapanema River, which corroborates the contributions of the Cinzas River basin for maintaining migratory fish populations in the Capivara Reservoir region. In addition, the present study provides additional evidence that the population dynamics of Neotropical potamodromous fish can vary between species and within species, in different scenarios, distancing itself in many cases from panmixia patterns, which reinforces the need for action of management and conservation that also include investigations of temporal genetic structure patterns, occurrence of homing behaviors, as well as possible factors that alter natural migration patterns.

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ETHICAL STATEMENT

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COMPETING INTERESTS

The author declares no competing interests.

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