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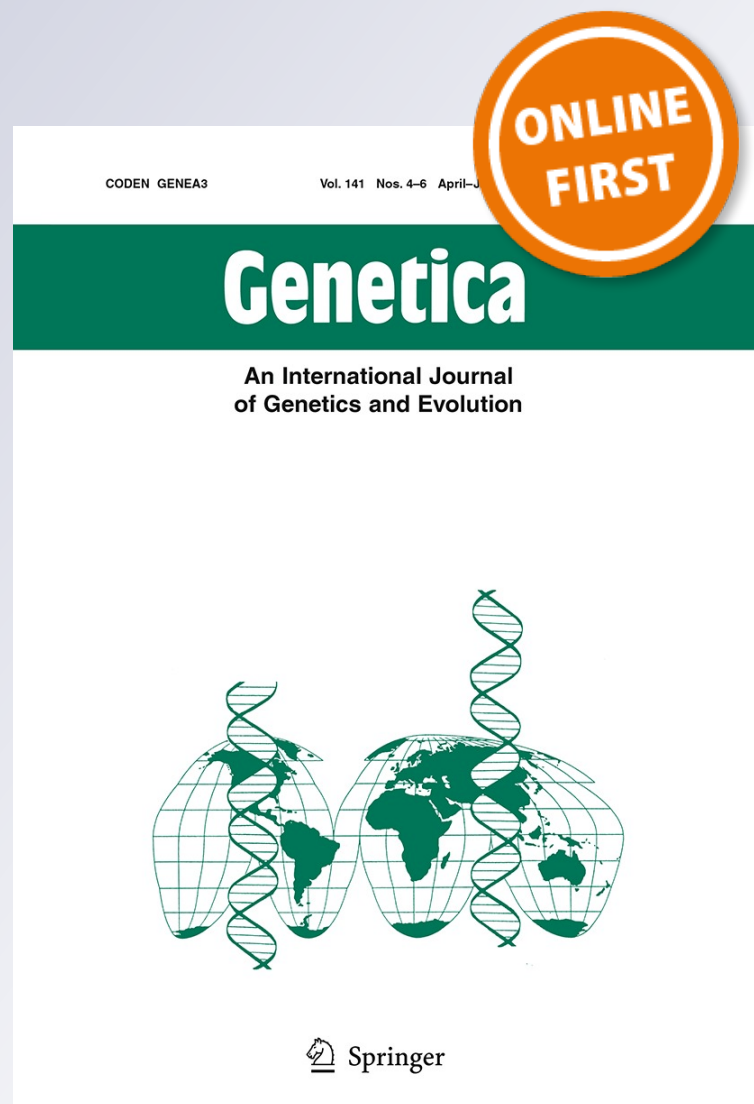
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Seasonal variation in genetic population structure of sábalo (*Prochilodus lineatus*) in the Lower Uruguay River

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Abstract *Prochilodus lineatus* is a highly migratory fish species that sustains the most important commercial fishery of Paraná-Paraguay basin. Migratory patterns are poorly known and only few population genetic studies are available for this species in the Upper Paraná. To assess genetic population structure, we genotyped a sample of 93 individuals from the Lower Uruguay River close to Gualaguaychú city (Entre Ríos, Argentina) at three different times, July 2008 (Winter), September 2008 (Spring) and

May 2009 (Fall). All individuals were genotyped for 12 microsatellite loci previously found to be informative to assess populations of *P. lineatus*. Our results show seasonal variation of the genetic sub-structuring at this locality that may be related to the presence of different migratory stocks throughout the year. The Fall sample includes an additional genetic cluster of individuals not detected in Winter and Spring, suggesting that this species should be considered a mixed stock fishery.

Keywords Microsatellites · *Prochilodus* · Population structure · Migratory fish · Paraná basin

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Introduction

Testing hypotheses of population structure is challenging in highly migratory species. Long-lived animals with seasonal migratory patterns may belong to discrete genetic stocks that overlap spatially for part of the year, potentially causing discordant patterns of population structure over time. Such is the case of mixed stock fisheries, whose populations consist of a variety of cohorts, sizes, and geographic or genetic stocks. Discrete populations of anadromous fishes such as salmonids converging on vast coastal marine areas during part of their life cycle are recognized as a classic example of mixed stock fisheries (Grant et al. 1980). Disentangling the mixture of distinct genetic pools converging in such areas is important for management and conservation, but requires powerful genetic markers and statistical approaches (e.g., Gauthier et al. 2013). Other well-characterized cases involve populations of marine organisms such as turtles (Bowen 1995; Naro-Maciel et al. 2007), but little is known about the incidence of mixed stocks and population structure in

migratory freshwater fish populations that inhabit large and complex Neotropical River systems.

Prochilodus lineatus (Valenciennes 1837) is the most abundant fish species in the vast Paraná-Paraguay River basin in South America (Fig. 1) in terms of absolute biomass (Sverlij et al. 1993). Commonly known as “sábalo” (in Argentina, Uruguay, Paraguay, Bolivia) or “curimbatá” (in Brazil), this species sustains important commercial and artisanal fisheries throughout its range. Large populations of prochilodontids feed on detritus, algae and associated microorganisms (Bowen 1983; Bowen and Bonetto 1984), and constitute an important link for energy and biomass transfer in riverine food webs (Winemiller 1996). The life cycle of prochilodontids includes conspicuous seasonal reproductive and feeding migrations (Barbarino Duque et al. 1998). Migratory patterns of *P. lineatus* have been described for most of the Paraná and Rio de La Plata basin on the basis of mark-recapture experiments, biotelemetry, or using micro data-recording tags, and elemental analyses of hard tissues such as the otoliths (Bayley 1973; Godinho and Kynard 2006; Lucas et al. 2001; Peixer and Petrer Junior 2009). A review of mark-recapture data (Sverlij et al. 1993) suggests that extensive reproductive migrations may occur, documented by specimens recaptured over 1000 km away from the tagging locality. But shorter migratory routes seem to be more likely, as indicated by the majority of recaptures between 50 and 500 km away from the marking location (Espinach Ros et al. 1990). Another study (Bonetto and Pignalberi 1964) reports fish tagged in the Rio de la Plata downstream of Buenos Aires recaptured in the Lower Uruguay River, some 250 km away, the following year. Other tagging experiments in the lower Uruguay and Rio de la Plata basins indicate upstream migrations along the Paraná River in the Fall, with almost no recaptures in the upper Uruguay (Espinach Ros et al. 1990; Bonetto et al. 1981). Relatively low fishing pressure in the upper and middle sections of the Uruguay River may explain the paucity of recaptures. This basin is characterized by short tributaries, normally interrupted by waterfalls, constraining migratory species and large fish to the main river channel and the lower section of the tributaries. The migratory species present in the Lower Uruguay River normally rely on floodplain lakes for larval and juvenile rearing. As these lakes are absent from the steep valleys of the Upper and Middle Uruguay, the species seem to use the mouths of tributaries as rearing areas (Zaniboni-Filho and Schulz 2003). The broad connection between the Uruguay basin with the Paraná River through the Rio de la Plata Estuary provides ample opportunities for dispersal of fishes among basins. A phylogeographic study of *P. lineatus* based on mitochondrial DNA (mtDNA) reported high levels of polymorphism but lack of population structure among broadly sampled individuals from the Parana-

Paraguay drainage, suggesting historically high levels of gene flow across the entire basin (Sivasundar et al. 2001). In spite of their commercial and ecological importance, migratory routes and population structure of *P. lineatus* in the Paraná-Paraguay basin are incompletely known.

Microsatellite markers have emerged as the most powerful and versatile source of information to assess population structure at finer levels, beyond the possibilities afforded by mtDNA (Goldstein and Schlötterer 1999). They are of particular interest to ecologists because they provide insight into fine-scale ecological questions (Selkoe and Toonen 2006). Combined with the Bayesian clustering approaches (e.g., Pritchard et al. 2000; Falush et al. 2003), these data can be used to estimate the number of distinct populations and to assign individuals to discrete migratory stocks. For example, studies conducted on the catfish *Pseudoplatystoma corruscans* showed that this species exhibits homing behavior towards exclusive tributaries in the upper Paraná-Paraguay basin in Brazil (Pereira et al. 2009). Another species in the same basin (*Piaractus mesopotamicus*) did not exhibit this behavior, but was shown to have marked genetic structure due to restricted gene flow (Calcagnotto and DeSalle 2009). The co-existence of different reproductive stocks of *Prochilodus argenteus* was reported inhabiting the area of Tres Marias dam on the São Francisco River in Brazil (Hatanaka et al. 2006). These studies show that much can be learned from microsatellite markers to detect distinct genetic stocks of migratory species. We use this approach to assess seasonal population structure of *P. lineatus* in the Lower Uruguay River, where large populations of this species congregate throughout the year and support a commercial fishery.

Materials and methods

Sampling and molecular methods

A total of 118 specimens were sampled from the Uruguay River close to city of Gualeguaychú (Entre Ríos, Argentina, 33° 6' 19.32" S; 58° 22' 10.14" W; Fig. 1). Samples were obtained on three different dates: July of 2008 (Winter, N = 48 individuals), October 2008 (Spring, N = 37), and April of 2009 (Fall N = 33). The specimens were caught along a range of 10 km upstream and 20 km downstream from Gualeguaychú using gill nets and seines. Specimens were euthanized and muscle tissue samples obtained were frozen immediately in liquid nitrogen for further analysis. Genomic DNA was extracted from muscle tissue using AccuPrep® Genomic DNA Extraction Kit.

Each individual was genotyped for 12 microsatellite loci, following procedures previously described (Rueda et al. 2011). These loci were shown to be selectively

Fig. 1 Geographic location of the study site along the Lower Uruguay River (black dot). Note ample connectivity of this site with the broader Paraná Basin (gray shading) through the Río de la Plata estuary



neutral and unlinked for *Prochilodus* populations. Microsatellite fragments were scored for length with an ABI 3730xl analyzer[®] (Applied Biosystems) using LIZ500 as the internal lane size standard. Fragment lengths were assigned to allelic classes with GENEMAPPER 3.1[®] (Applied Biosystems).

Statistical analysis

We first tested the occurrence of genotyping errors that may result from PCR artifacts (as null alleles) or low DNA quality (large allele dropouts), and for Hardy–Weinberg equilibrium (HWE) using MICRO-CHECKER (van Oosterhout et al. 2004). To estimate basic descriptive parameters such as the observed and expected heterozygosities, allele number, and gene diversity values for each sample and locus we used ARLEQUIN v.3.5.1.2 (Excoffier and Lischer 2010) and FSTAT v. 2.9.3.2 (Goudet 1995) for allele richness values. We implemented non-parametric Wilcoxon tests to determine if differences observed in

expected heterozygosity among samples were statistically significant using the program PASW STATISTICS 16 (Norusis 2008). Next, to gauge genetic differentiation among seasonal samples (Winter, Spring, and Fall) we used pairwise F_{ST} with routines implemented in ARLEQUIN v. 3.5.1.2. Finally, to assess population structure as indicated by the number of genetically distinct populations (clusters) that may be present at the sampling location throughout the year we used the Bayesian clustering approach implemented in STRUCTURE (Pritchard et al. 2000; Falush et al. 2003). This method calculates posterior probabilities for k clusters, assuming Hardy–Weinberg equilibrium conditions within clusters, while minimizing linkage disequilibrium. Runs were performed using a burn-in of 150,000 iterations followed by a Markov chain Monte–Carlo of 300,000 steps. Analyses were performed on the complete data set for the study system. For both analyses, average posterior probabilities were calculated from 10 independent runs, for k ranging from 1 to 10 to determine the optimal value of k , following recommendations by

Table 1 Summary statistics of allelic variation at 12 microsatellite loci for *P. lineatus*

Locus	N	Allele number	Allele richness	Gene diversity
PL3	93	12	11.46	0.80
PL14	91	19	16.83	0.85
PL23	70	7	6.71	0.23
PL25	91	23	19.67	0.89
PL28	69	3	3.00	0.28
PL34	91	15	12.77	0.71
PL35	61	24	24.00	0.90
PL64	86	22	20.96	0.93
PL190	76	19	18.40	0.91
PL119	87	31	28.53	0.95
PL139	79	40	36.81	0.96
PL216	91	10	8.34	0.20

Evanno et al. (2005). We chose the “admixture model” and the option of correlated allele frequencies between groups, as this configuration is considered best by the authors of this method (Falush et al. 2007).

Results

All 118 individuals were genotyped for 12 loci, but 25 samples for which less than 10 loci could be determined due to PCR failure were excluded from further analysis. The genetic data set for analysis was therefore reduced to $N = 31$ individuals out of 48 for the Winter sample, $N = 29$ out of 37 (Spring), and $N = 33$ out of 33 (Fall). Two loci had low amplification efficiency (PL35: 66 %; PL28: 74 %; Table 1) but they did not show signs of null alleles according to Microchecker. For all loci and individuals combined, Microchecker results suggest that this population is possibly in Hardy–Weinberg equilibrium with loci PL14, PL64, PL119, PL139, PL216, showing signs of a null allele, as is suggested by the general excess of homozygotes for most allele size classes. Given that subpopulation structure was diagnosed by STRUCTURE analysis (below), it is possible that the detected reduction of heterozygosity may be due to Wahlund effects. No evidence of allele dropout or genotyping errors due to stuttering was found. Genetic diversity at each locus (equivalent to expected heterozygosity) ranged between 0.198 and 0.930, the number of alleles observed per locus between 3 and 40, and allele richness per locus estimated on a minimum sample size of 61 individuals was between 3.0 and 36.8 (Table 1). Average observed heterozygosity values over all loci $H_O = 0.63$ (SD 0.25) while expected heterozygosity $H_E = 0.71$ (SD 0.29). These results confirm

Table 2 Genetic diversity for each seasonal sample

	Fall (n = 33)	Winter (n = 31)	Spring (n = 29)
AN	Total = 132 Range 2–23 Avg = 13.2 (± 6.6)	Total = 131 Range 4–24 Avg = 13.1 (± 6.5)	Total = 141 Range 3–27 Avg = 14.1 (± 7.4)
H_E	0.78 (± 0.28)	0.77 (± 0.20)	0.81 (± 0.20)
H_O	0.69 (± 0.26)	0.73 (± 0.21)	0.71 (± 0.20)
AG	0.82 (± 0.45)	0.461 (± 0.36)	0.83 (± 0.45)

Standard deviations are given in parentheses

AN allele number, Avg average, H_E expected heterozygosity, H_O observed heterozygosity, AG average gene diversity over all loci

that the microsatellite data obtained are valid and informative for population genetic analysis.

Population structure

Comparison of samples taken at different times of the year shows that the genetic signature of the sábalo population in the Lower Uruguay River around Gualeguaychú varies significantly throughout the year. Diversity values for the three seasonal samples (mean of allele number, gene diversity, observed and expected heterozygosity over all loci) are shown in Table 2. The non-parametric Wilcoxon test shows that the differences between H_E values obtained were significant (with $P < 0.05$). Inspection of pairwise F_{ST} values suggests that there is no genetic differentiation between samples taken in Winter and Spring ($F_{ST} = 0.0061$) but that the Fall sample exhibits a major shift in genetic composition. Pairwise F_{ST} values involving the Fall sample are at least 20 times higher: Fall–Winter $F_{ST} = 0.141$, and Fall–Spring $F_{ST} = 0.126$.

STRUCTURE analyses indicated that the most probable number of clusters in the total sample was $k = 3$ (Fig. 2a). Consistent with pairwise F_{ST} values reported above, STRUCTURE also finds that the Fall sample is different from Winter and Spring because it contains many individuals that are assigned to group 3 (black bars) with highest probability, whereas this group is conspicuously absent in Winter and Spring samples. (Fig. 2b). Taken together, these analysis strongly suggest that a distinct genetic stock of *P. lineatus* enters this locality in the early Fall (or during the Summer, for which we have no samples) and that the Winter and Spring populations contain at least two distinct stocks.

Discussion

Microsatellite makers used in this study exhibit a high number of alleles, ranging from 3 to 31, indicating that they

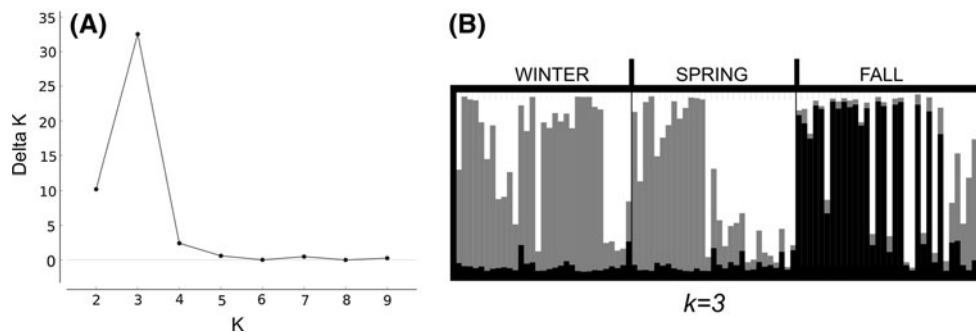


Fig. 2 Results of genetic analyses of microsatellite data for *Prochilodus lineatus* from Lower Uruguay River using STRUCTURE. Individuals are grouped by sampling season (Winter, Spring, Fall). **a** Plot of mean Delta K obtained using Structure Harvester (Earl and vonHoldt 2011) for 10 independent runs varying the k parameter

from 1 to 10, showing that $k = 3$ has the highest probability. **b** Assignment probabilities for each individual (vertical bars) into one of three clusters. White and gray bars represent genetic clusters 1 and 2, respectively. Cluster 3 (black bars) is only present in the Fall sample

are a potentially informative tool for population genetics analysis of this species (Rueda et al. 2011; Kalinowski 2004). Previous analyses of *P. lineatus* mtDNA (Sivasundar et al. 2001) reported high levels of genetic variability and gene flow but no genetic structure across the Paraná basin. In contrast, our analysis of microsatellite data for a single locality on the Uruguay River demonstrated the presence of up to three distinct genetic stocks, highlighting the well-known power of these markers to detect genetic structure (Goldstein and Schlötterer 1999; Waples 1998).

Correlations among population structure and dispersal rate or high gene flow have been shown in many marine fish species with broad geographic distribution patterns (Schunter et al. 2011; Wirth and Bernatchez 2001; Dannewitz et al. 2005; Jørgensen et al. 2005). Also well explored are freshwater species such as salmonids and whitefish that inhabit rivers and large postglacial lakes in North America (e.g., Fraser and Bernatchez 2008; Bernatchez and Wilson 1998). In contrast, little is known about population structure and phylogeography in the Southern Hemisphere (Beheregaray 2008), especially among highly migratory fishes living in large Neotropical River systems.

Prochilodus lineatus is known to exhibit complex migration patterns throughout the entire Paraná-Paraguay River basin (reviewed by Sverlij et al. 1993). Their migrations have been characterized by capture–recapture experiments in the Paraná and Uruguay Rivers, and are consistent with well-known reproductive behaviors tied to annual flood pulses (Lowe-McConnel 1975). When river discharges increase at the onset of the rainy season (October to January, depending on latitude), upstream migrating adult fishes move towards spawning grounds to mate in open waters of the main channel (Stassen et al. 2010; Espinach Ros et al. 1990). A consequence of this spawning behavior is that eggs and developing larvae are rapidly transported downstream towards the floodplain,

where they are distributed by the rising waters over recently inundated feeding grounds (de Yuan 1992; Araujo Lima and Oliveira 1998; Welcomme et al. 2006). After spawning, adult fish move downstream towards the Río de La Plata during the Spring, where they find abundant detritus along shallow backwaters during the Summer, until late February or early March. Towards the end of Summer they start upstream migrations along the Paraná and Uruguay River (Quirós and Cuch 1989). This pattern is consistent with our results, since sábalo captured in the Lower Uruguay in the Fall carry a different genetic signature than fish captured in this area during Winter and Spring. In contrast to the Uruguay River, *P. lineatus* in the Paraguay and Paraná basins move upstream from September to October reaching the headwaters close to the spawning area by the end of the dry season. This difference in behavior may reflect the lack of a well-defined dry season in the Upper Uruguay—otherwise typical of the Upper Paraná-Paraguay basin—where rainfall is more evenly distributed throughout the year causing only minor floods, also constrained by deeper river valleys and the absence of marginal lakes and floodplains. Other studies suggest that in the Uruguay basin groups of migrating sábalo may be moving in opposite directions at the same time (Espinach Ros and Rios 1997), or that populations of migratory fish in the lower Uruguay find their spawning grounds in the middle Paraná River (Sverlij et al. 1998). Regardless of the specific migratory pattern, genetically differentiated stocks moving up and down the river certainly may have a profound effect on the genetic structure of populations assayed in a single location at different times of the year. The co-existence of genetically different reproductive stocks of *P. argenteus* in the São Francisco River (Hatanaka et al. 2006) points to this possibility for *Prochilodus* species. In this study we show that this is the case for populations near Gualaguaychú along the Lower Uruguay River. Other

documented examples of seasonal variation in population structure include the Nile tilapia *Oreochromis niloticus*, (Bezault et al. 2011).

Our results suggest that the Lower Uruguay River may act as a “transit area,” where different genetic stocks arrive to feed and then move downstream to the Río de La Plata or to the channels of the vast Paraná delta to eventually reach the middle Paraná spawning grounds to reproduce. Treatment of *Prochilodus* commercial resources as a mixed stock fishery may lead to more desirable results for wildlife managers than the common practice so far to consider it a single panmictic population. Assessment of seasonal variation in population structure could be extended productively to other river systems (Paraná, Paraguay, Pilcomayo and Río de La Plata) to infer an integrated migratory pattern for *P. lineatus* over the entire Paraná-Paraguay Basin.

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