


Preliminary insights into the genetic mating system of Neotropical *Salminus brasiliensis*: kinship assignment and parental reconstruction reveal polygynandry

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Abstract Many relevant aspects of the breeding ecology of South American migratory fishes remain obscure. We conducted kinship analyses and parental reconstruction based on microsatellite genotypes of 41 *Salminus brasiliensis* larvae sampled from a single natural spawning event. Polygynandry involving 25 (12:13) potential parents was suggested, supporting ecological observations of aggregation during spawning. Some parents contributed up to five half-sibling larvae genotypes, implying a skewed contribution of genes to the progeny. This study

contributed to preliminary insights into the *S. brasiliensis* genetic mating system.

Keywords Freshwater fish spawning · Fish reproduction · Molecular markers · Multiple paternity · Polygamy

Introduction

The reproductive behavior has important implications for the ecology and genetic structure of populations. Breeding strategies vary greatly in nature, and fishes are not an exception to this (Avisé et al. 2002). Neotropical migratory fishes usually have small oocytes and non-adhesive eggs, and show high fecundity and lack of parental care (Vazzoler 1996; Godinho et al. 2010). Many relevant aspects of the reproductive biology of Neotropical migratory fishes in nature—such as, for example, how many breeders participate in a spawning event, whether they establish territories or monopolize reproduction—still remain obscure, and South American species continue to be understudied (Reynalte-Tataje et al. 2012). The genetic mating system is derived from the reproductive tactics that male and females use to contribute their genes to a progeny and is often very difficult to determine from field observations alone (Avisé et al. 2002; Xue et al. 2014). Nowadays, modern kinship analyses coupled with reconstruction of parental genotypes based on molecular markers allow inference of the genetic mating system in several fish species, even when adult breeders cannot be sampled (e.g., Kanno et al. 2011; Rodriguez-Barreto et al. 2013).

To contribute to the better understanding of the genetic mating system of Neotropical migratory freshwater fishes, we focused on *Salminus brasiliensis* (Characiformes: Characidae). This species inhabits the southern portion of

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the South American continent, reproduces annually and shows total spawning (Lowe-McConnell 1999). Fertilization occurs in the water and there is no parental care (Lowe-McConnell 1999; Sato and Godinho 2003). Such a reproductive behavior makes it difficult to disentangle the number of parents that actually sire a progeny in nature. To gain preliminary insights into the genetic mating system of *S. brasiliensis* in the wild, we used molecular markers to assess kinship patterns among larvae from a natural spawning event, and reconstructed parental genotypes to determine the number of different adults that may have taken part in reproduction.

Materials and methods

Sampling was conducted in January 2010 at the mouth of the Ligeiro River (27°31' S; 51°50' W)—a small tributary were fishes spawn—in the Uruguay River basin, Southern Brazil [Electronic Supplementary Materials (ESM) Fig. S1]. Eggs were sampled using plankton nets and following Hermes-Silva et al. (2009). As *Salminus brasiliensis* hatch about 15 hours after fertilization (Zaniboni-Filho and Nuñez 2008), we are confident that all the eggs were from a single spawning event. Eggs were immediately incubated and, after hatching, larvae were preserved in 96 % ethanol. Species identification was carried out by an expert taxonomist, following Nakatani et al. (2001) and Reynalte-Tataje and Zaniboni-Filho (2008).

Genomic DNA was isolated from each of 41 *S. brasiliensis* larvae using salt extraction (Aljanabi and Martinez 1997). Eight heterologous microsatellites (Table 1) were amplified by polymerase chain reaction; cycling protocols and genotyping methodology followed Rossini et al. (2011). Genotypes were inspected for null alleles, stuttering and dropout with MICRO-CHECKER v2.2.3 (van Oosterhout et al. 2004). To evaluate the power of the set of microsatellites for subsequent kinship analyses and parental reconstruction, standard indices of genetic diversity (Table 1), including the probability of identity (PI) and of exclusion (PE) were computed from a sample of 53 *S. brasiliensis* adults collected at the Upper Uruguay River. We computed mean relatedness among adults (R , Queller and Goodnight 1989) and verified that they were unrelated ($R = -0.022$). Computations were performed on GENALEX v6.5 (Peakall and Smouse 2012) and GENEPOP v.1.2 (Raymond and Rousset 1995).

Genotypes of the unsampled *S. brasiliensis* adults that may have contributed to the progeny of larvae were reconstructed using COLONY v.2.0 (Wang and Santure 2009; Jones and Wang 2010). The program COLONY uses a full maximum-likelihood method based on multilocus data to

Table 1 Summary statistics of genetic diversity for microsatellite loci amplified in *Salminus brasiliensis* adults collected in the Upper Uruguay River, Brazil

| Locus | n | A | Ae | Ho | He | F_{is} | $pHWE$ |
|---------------------|----|----|-------|------|------|----------|--------|
| Sfra02 ^a | 52 | 31 | 23.41 | 0.87 | 0.96 | 0.10 | 0.01 |
| Sfra03 ^a | 53 | 15 | 6.78 | 0.70 | 0.85 | 0.18 | 0.34 |
| Sfra04 ^a | 52 | 30 | 21.46 | 1.00 | 0.95 | -0.05 | 0.06 |
| Sfra10 ^a | 53 | 15 | 5.98 | 0.93 | 0.83 | -0.11 | 0.00* |
| Sfra13 ^a | 53 | 11 | 4.66 | 0.81 | 0.79 | -0.03 | 0.41 |
| Sfra18 ^a | 52 | 6 | 2.81 | 0.65 | 0.64 | -0.02 | 0.71 |
| Sfra14 ^a | 47 | 27 | 14.44 | 0.94 | 0.93 | -0.01 | 0.10 |
| Sh05 ^b | 33 | 19 | 10.37 | 0.91 | 0.90 | -0.01 | 0.73 |
| Overall loci | 49 | 19 | 11.24 | 0.85 | 0.85 | 0.01 | 0.34 |

Number of individuals genotyped (n), number of alleles (A), effective number of alleles (Ae), observed (Ho) and expected (He) heterozygosity, inbreeding coefficient (F_{is}) and probability of deviation from Hardy–Weinberg equilibrium ($pHWE$). The asterisk denotes a significant p value after Bonferroni correction. Primer references (a and b) are given in Rossini et al. (2011) and Silva and Hilsdorf (2011), respectively

simultaneously infer parentage and sibship. It reconstructs genotypes of candidate parents based on allelic information from individuals, which form sib groups. COLONY infers maternal or paternal groups even without parental information, although we cannot determine which of the two inferred groups is mother or father (e.g., de Mestral et al. 2012). This method was developed to be applied to cases in which both sexes can be polygamous, and it can be used to infer fullships, paternal and maternal half-sibships, as well as parentage jointly with sibships based on the Mendelian law of inheritance, allele frequencies, and Hardy–Weinberg equilibrium. This statistical framework is appropriate for a polygamous mating system in which a cohort of larvae can include unrelated (UR) individuals, as well as full siblings (FS) and half-siblings (HS) (Emery et al. 2001). This approach in COLONY can be applied on the genetic mating system without parental samples (e.g., Rodriguez-Barreto et al. 2013). In this study, parameters for the COLONY analyses were: very high likelihood precision, random seed, without candidate males, without candidate females and polygamy for both sexes. To minimize bias in kinship analyses, the allele frequencies computed for the 53 adults were entered as input into COLONY. To further check parental reconstruction and clustering of larvae into sib groups made by COLONY, we employed the Web-based application PEDIGREE v 2.0 (available at: <http://herbinger.biology.dal.ca:5080/Pedigree>) which uses a Markov Chain Monte Carlo (MCMC) algorithm to carry out those tasks. In addition, we computed relatedness between larvae pairs and checked if the sib groups clustered by COLONY and PEDIGREE agreed with the relationship category (UR, HS or FS) inferred from R .

Results

The microsatellites used were highly polymorphic, with six to 31 alleles/loci. There was no evidence of linkage disequilibrium for any pair of loci, no evidence of inbreeding, and only Sfr10 showed deviation from Hardy–Weinberg equilibrium (Table 1). The expected heterozygosity ranged from 0.64 to 0.96 (Table 1). The combined *PE* for the eight loci was 0.99 and the *PI* was 3×10^{-12} .

Kinship patterns and parental assignment identified 25 potential parents (12:13). Although we cannot determine which is the maternal or paternal group for the assigned groups (i.e., 12 and 13 individuals), clear tendencies of polygynandry and reproductive skew were observed. Parental inference indicated that most individuals of one sex mated with two to five different partners, and most individuals of the opposite sex also mated with two to five partners (Table 2). One of the candidate parents produced eight larvae with five different partners, whereas others produced five larvae with multiple partners. From the inferred parental genotypes and mating, according to the sharing of one, two or zero parents, larvae could either be full siblings, half-siblings or unrelated (Table 2).

The classification of larvae into sibship groups made by COLONY and PEDIGREE found 54 supposedly related pairs according to mating inference, and 90.75 % were correctly classified by the *R*-value criterion computed with KINGROUP program (ESM Table S1). From these, nine larva pairs were inferred as full siblings (FS), 44 as half-siblings (HS) and one as unrelated (UR) (ESM Table S1). An example of misclassification is pair L02–L36, which would be half-siblings according to COLONY, but were identified as FS by the *R*-value criterion (Table S1). Moreover, all of the 61 larvae pairs that did not share any parents according to COLONY or PEDIGREE reconstructions were confirmed to be unrelated by the *R*-value criterion (data not shown).

Discussion

The present study contributes novel insights into the mating ecology and genetic mating system of the Neotropical *Salminus brasiliensis* and constitutes the first report of polygamy in a South American migratory fish. The genetic mating system of *S. brasiliensis* was deduced from sibship patterns among larvae inferred from molecular marker-based analyses, and from the number of parents necessary to produce the observed progeny genotypes. In theory, depending on the mating system of the species under study, a group of larvae collected in a natural spawning event can either represent a large number of parents, yielding families

Table 2 Families inferred for 41 *Salminus brasiliensis* larvae collected at the Ligeiro River, Brazil. Parental groups were inferred by a maximum-likelihood approach in COLONY v2.0 (Jones and Wang 2010), and confirmed by a Markov Chain Monte Carlo approach in PEDIGREE v.2.0 (Herbinger et al. 2006), based on progeny genotypes using eight microsatellites. We assigned one of the parental sexual groups as “Putative males” and the other as “Putative females” for convenience, although this may be opposite (details in the text)

| Family group | Putative males | Putative females | Larvae |
|--------------|----------------|------------------|--------|
| 1 | *1 | #1 | L1 |
| | | #2 | L32 |
| | | #4 | L4 |
| | | #9 | L40 |
| 2 | *2 | #2 | L2 |
| | | #7 | L36 |
| | | #3 | L42 |
| | | #6 | L6 |
| 3 | *3 | #6 | L29 |
| | | #3 | L3 |
| | | #7 | L31 |
| 4 | *4 | #4 | L15 |
| | | #4 | L20 |
| | | #4 | L41 |
| | | #4 | L7 |
| | | #5 | L5 |
| 5 | *5 | #7 | L19 |
| | | #7 | L8 |
| | | #11 | L23 |
| | | #4 | L46 |
| 6 | *6 | #6 | L12 |
| | | #1 | L13 |
| | | #4 | L14 |
| | | #7 | L43 |
| | | #8 | L9 |
| | | #9 | L10 |
| 7 | *7 | #2 | L38 |
| | | #8 | L11 |
| 8 | *8 | #8 | L22 |
| | | #10 | L16 |
| 9 | *9 | #4 | L17 |
| | | #12 | L25 |
| | | #12 | L44 |
| 10 | *10 | #3 | L27 |
| | | #6 | L35 |
| | | #6 | L26 |
| | | #5 | L28 |
| 11 | *11 | #1 | L30 |
| | | #3 | L33 |
| | | #11 | L39 |
| 12 | *12 | #3 | L33 |
| | | #11 | L39 |
| 13 | *13 | #12 | L37 |
| | | #12 | L37 |

with few related larvae, or a few parents, resulting in a large number of full- and half-sib larvae. Our results indicate aggregation of breeders during spawning and polygynandry, with multiple males and females participating in reproduction. Because around 80 % of the progeny was represented by half-siblings, it suggests that there was a skewed contribution of some individuals to the offspring's gene pool.

Although not exempt from limitations due to the low sample size used (41 larvae from a single natural spawning event), we are confident that the methodological approach employed here provides a fairly likely picture of *S. brasiliensis* mating in nature (see below). When inferring parentage and individual contribution to the progeny, the use of highly polymorphic markers is very important (Xue et al. 2014). We used markers with up to 31 alleles per locus and a very high combined exclusionary probability (>99 %), sufficient to confidently reconstruct parentage. Moreover, the use of methods with different statistical frameworks, which highly agreed in their parental reconstruction and kinship clustering of larvae, minimized the chance of getting false results. Yet, to improve the genetic characterization of the *S. brasiliensis* mating system, future studies should sample several spawning events along the entire distribution range of this fish and, if possible, capture adult breeders that could be contributing to the species gene pool.

We identified 25 (12:13) potential parent *S. brasiliensis* that would have aggregated during spawning and taken part in fertilization. This finding provides preliminary genetic support for the field observation of single *S. brasiliensis* females accompanied by many males during spawning (Godinho et al. 2010). Fish aggregation and formation of schools during the reproductive period are common in Neotropical migratory fishes (Sanches and Galetti 2012). Polygamous mating systems have been detected in other species, such as Atlantic salmon *Salmo salar* (see de Mestral et al. 2012) and the headwater brook trout *Salvelinus fontinalis* (see Kanno et al. 2011). Polygamy can allow reproduction of subordinate individuals, which otherwise would not take place in matings. For example, in most instances, smaller males will have limited access to females and polygyny can help increase males' fitness by allowing paternity of eggs from multiple females (Coleman and Jones 2011). Similarly, polyandry can indirectly augment females' fitness by increasing the genetic variability of the progeny (Coleman and Jones 2011), likely improving offspring survival and future reproduction. In species with total spawning, as *S. brasiliensis*, fertilization of all oocytes with semen of a unique suboptimal partner could compromise the survival of offspring, seriously affecting individual fitness and population dynamics in the long term. Therefore, polygynandry would represent a good reproductive strategy for this species.

Parental inference and sibship groups of *S. brasiliensis* were estimated through the congruent results obtained by

both COLONY and PEDIGREE programs, which can reconstruct pedigrees using microsatellite markers without parental information (Herbinger et al. 2006). Specifically, the COLONY software inferred the genotypes of the parental group that contributed to the progeny of larvae, although it could not accurately assign sex to a given genotype. Despite this limitation, our analyses still unraveled a skewed reproductive success of *Salminus brasiliensis* adults, with some of them mating with up to five mates (Table 2). This is not surprising as, in fishes, fecundity increases with body size, and larger females can produce more eggs (Vazzoler 1996). Similarly, larger males usually gain proximity to females during gamete release and can fertilize eggs of several females (Vazzoler 1996). Such a skewed contribution of genes to the next generation can, in the long term, lower effective population size (N_e) through increased variance in reproductive success and family size (Plesnar-Bielak et al. 2013). If this trend is maintained in *S. brasiliensis*, it could exert a particularly negative effect in genetic variability, precluding adaptation in face of the many human-induced threats that the species suffers. The main pressures on this important fisheries resource are those derived by the building of dams for hydroelectric power plants, which often represent barriers to fish dispersal and could compromise ongoing gene flow (Agostinho et al. 2004). Genetic monitoring through assessing variability, further characterizing mating system and estimating N_e should be part of future studies in *S. brasiliensis*. The baseline genetic methods and analyses used here can guide further research in this and similar fish species in the wild.

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