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Genetic evidence of unisexual reproduction in the Moroccan hexaploid barbel *Labeobarbus fritschi*

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Abstract. The hexaploid barbel population of the species *Labeobarbus fritschi* which lives in the Allal El Fassi dam reservoir in the north of Morocco showed an absence of males among a sample of 597 individuals analyzed in a first survey and among the 39 fish analyzed in this study. A possible explanation is that this population may be composed of females only and that they reproduce either by gynogenesis or hybridogenesis, two processes triggered by hybridization with a sexually incompatible species (possibly the sympatric barbel *Luciobarbus setivimensis* in this case). We used molecular markers to collect any information that could explain the phenomenon. To do this, numerous nuclear loci were screened (more than 50) in search of polymorphism. Genetic diversity was low as a possible consequence of clonal reproduction: only three allozymic and two intronic loci presented a sufficient level of polymorphism to be exploited. The existence of several copies of the same multilocus genotypes (1/3 of the fish) – with some showing a significant probability of not being derived from sexual reproduction – provided evidence of unisexual reproduction. Deviations from panmixia as well as linkage equilibrium were also observed. Although preliminary, the data supports the hypothesis of the unisexual reproduction of females by way of gynogenesis rather than hybridogenesis.

Key words: Morroco, allozymes, introns, gynogenesis, hybridogenesis

Introduction

The genus *Barbus sensu lato* (Cuvier & Cloquet, 1816) belongs to the family Cyprinidae, composed of more than 800 species (Howes 1987). This genus, in its *sensu lato* (*s.l.*) acceptation, gathers taxa from Asia, Europe and Africa; it is only diagnosed as possessing two pairs of barbels, with several exceptions (one pair or no barbels).

In this enormous aggregation (Myers 1960), recent taxonomic improvements based on molecular/cytogenetic data made it possible to identify large ploidy lineages already considered as distinct genera (Berrebi 1995, Berrebi et al. 1996, Skelton 2002, Berrebi & Tsigenopoulos 2002, 2003, Berrebi et al. 2013).

Labeobarbus fritschi (Günther, 1874) is a hexaploid barbel (El Gharbi 1994, Machordom & Doadrio 2001). Since 1996, the subgenus *Labeobarbus* has become a genus. It is widespread in Africa (Golubtsov & Krysanov 1993, Berrebi et al. 1996, Skelton 2002), particularly in Morocco (Machordom & Doadrio 2001) and in the Middle East (Durand et al. 2002, Tsigenopoulos et al. 2002). *L. fritschi* lives, among others, in the Allal El Fassi dam lake (Morocco), in the River Zerouka, a tributary of the River Sebu, north of Rabat (Bouhbouh 2002) where it accounts for most of the ichthyofauna with another tetraploid barbel, *Luciobarbus setivimensis* (Valencia, 1842).

Bouhbouh (2002) reported that samples of *L. fritschi* caught in this dam lake included only females (597 females from 597 fish sampled). The absence of males may be explained by two assumptions: (i) the males of this species were absent from the fishing sites since the barbel has a tendency to migrate, (ii) it is a monosex population consisting only of females and the reproductive process behind this phenomenon must be elucidated.

Teleost fish can develop different modes of reproduction including various forms of unisexual reproductions. Several mechanisms were described as self-fecundation in some hermaphrodites, or gynogenesis and hybridogenesis in unisexual females of some populations (Chevassus 1998, Baroiller & Guiguen 2001, Gui & Zhou 2010, Collares-Pereira et al. 2013).

Based on the genetic analysis of barbel using nuclear molecular markers, the objective of this study is to understand the relevance of each one of the two

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hypotheses: differential migrations between the sexes, or a unisexual population reproducing either by gynogenesis or hybridogenesis.

Material and Methods

Thirty-nine individuals of the species *L. fritschi* were collected in February 2002 using gillnets in the Allal El Fassi reservoir. The fish were frozen at –20 °C immediately after capture and transported to the laboratory in Montpellier, France. The sex of each of the 39 fish was verified by observation of gonads with a microscope after dissection. The molecular markers analyzed were of two types: allozymes and introns (EPIC – Exon Primed Intron-Crossing).

Allozymes

The enzyme extraction was performed on muscle and liver tissue of each fish according to the protocols described by Pasteur et al. (1987). The desired enzyme loci were highlighted by colour-adapted protocols by Berrebi et al. (1993, 1995) and Tsigenopoulos et al. (1999). Ten enzyme systems (IDH, LDH, PGI, MDH, 6PGD, AAT, GDA, AK and PGM) and the soluble muscle proteins (PT), corresponding to 25 presumptive loci, were tested. Table 1a presents details of the retained loci and buffers used (nomenclature according to Shaklee et al. 1990).

Introns

DNA was extracted according to the classical phenol/chloroform protocol (Sambrook et al. 1989). EPIC-PCR used primers complementary to exon sequences flanking introns. Exon sequences are mostly conserved among the species, providing universal priming zones which hold a practical interest (Atarhouch et al. 2003). A total of 27 primer pairs were tested in this study. Table 1 lists the characteristics of the retained loci for the analysis. PCR reactions were performed using an Eppendorf Mastercycler in a volume of 10 µL for each individual, containing 1 µL of 10X buffer (Promega), 2.5 mM MgCl 2, 0.2 mM of each dNTP (Invitrogen), 0.5 µM of each of the two primers, one of which is labeled with a fluorochrome (Cy3, Cy5 or Fuorescein, MWG-Biotech), 0.3 U of *Taq* polymerase (Sigma) and 1 µL of DNA template at about 150 µg/mL.

The PCR program included an initial denaturation at 94 °C for 3 min, then 35 cycles consisting of denaturation for 1 min, binding of the primers (annealing) at the temperature specific to each locus (see Table 1b) for 1 min and extension at 72 °C for 1 min 20 sec; the program ended with a final extension at 72 °C for 10 min.

The PCR products were then subjected to electrophoresis on acrylamide denaturing (urea) 8 % gel. This gel was read by a Hitachi FMBIO II scanner. The FMBIO Analysis 8.0 software can accurately measure allele sizes in number of base pairs (bp).

Statistical analyses

The data matrix was statistically processed involving three steps:

1) Calculation of the parameters commonly used in population genetics (allele frequencies, heterozygosity).

Table 1. Marker descriptions.

a) Allozymic loci: tissue of extraction, electrophoretic buffers and staining solution compositions.

Enzymatic loci				
Locus	Tissue	Gem buffer	Electrode buffer	Staining
PGI-2*	liver	TCB 8.7	Borate 8.2	5 mL tris Hcl 0.2M ph 8 1 mL Mgcl2 0.5M 1 mL NAD 0.5 mL NADP 10 mg fructose 6 P 0.5 mL PMS 0.5 mL MTT 1 mL NBT 6 µL G6PDH Agar
PGI-3*	liver	TCB 8.7	Borate 8.2	
PGI-4*	muscle	Tris HCl	Borate 8.2	

b) Intronic loci: primer sequences (F = forward, R = reverse), original publication and specific annealing temperature.

Intronic loci			
Locus	Primers	References	Tm
RP	F = TGGCCTCTTCCTTGGCCGTC R = AACTCGTCTGGCTTTTCGCC	Chow & Takeyama 1998	54 °C
Mlc2a	F = CAGCTGTCCCATGGTGGCCA R = GGCCGGTACCTCCAATGTGTTC	Atarhouch et al. 2003	55 °C

2) Calculation of Wright's fixation indices such as Fis (checking panmixia) by the estimator f of Weir & Cockerham (1984) and linkage disequilibrium (Black & Krafsur 1985). The statistical significance of these parameters was estimated by resampling permutations tests (5000 permutations). The GENETIX software (Belkhir et al. 1998) was used for these first two steps.
3) The clonality of genotypes was tested using the GENECLONE software (Arnaud-Haond & Belkhir 2007). This software established the probability of existence for each multilocus genotype taking into account the Fis of the population analyzed, i.e. a deviation from panmixia as described by Young et al. (2002). Next, the probability that n repetitions of the same genotype can occur in the population by n independent events of sexual reproduction was estimated (psex; Tibayrenc et al. 1990, Parks & Werth 1993, Arnaud-Haond et al. 2005). If psex < 0.05, repeated genotypes have a significant probability of being propagated by unisexual reproduction.

Results

Dissection and observation under a binocular microscope of a gonad for each of the 39 fish revealed that they were all females, the gonads containing oocytes that were easily recognizable, at different stages of maturity between individuals.

Among the 10 enzymatic systems tested, 5 showed interpretable zymograms: AK, PGI, MDH, PGM and PT despite hexaploidy, for a total of 8 loci. The loci AK*, MDH-1* and MDH-2* were monomorphic in the sample and uninformative. The loci PT* and PGM* showed a presence/absence polymorphism not used for our demonstration.

Only three loci of the PGI system (Phospho-Gluco-isomerase) were polymorphic and usable for our purpose: the loci PGI-2* and PGI-3* migrated to the cathode while PGI-4* migrated to the anode.

Among the twenty-seven intronic systems tested, only two gave usable results with a measurable length polymorphism: the PR and Mlc2a systems. Each had only a single polymorphic locus, in both cases with two alleles. Other systems tested were either unreadable due to overlapping bands (12) or monomorphic (13).

Enzymes and introns data were gathered in a single matrix which was subjected to the statistical analyzes described above.

1) Table 2 gives the allelic frequencies of enzyme loci and introns, as well as the partial estimated (considering only the polymorphic loci) and observed heterozygosities.

2) The estimate of Fis produces a negative value (–0.328) that is highly significant ($p < 0.001$). This negative value indicates an excess of heterozygotes (see Table 2) and thus a deviation from Hardy-Weinberg equilibrium (HWE) in the barbel population. Similarly, the test of linkage disequilibrium between the four loci shows that the pairs of loci PGI-2*/Mlc2a and PGI-4*/RP were significantly correlated ($p < 0.001$).

3) The test of clonality was applied to the data set. A group of multi-locus genotypes (those of individuals h4, h14, h25 and h36, see Appendix) showed a significant probability (psex < 0.03) of not having been generated by sexual reproduction and therefore generated by clonal reproduction.

Table 2. Allelic frequencies and heterozygosities of the five loci analyzed. Ho = observed heterozygosity, He = expected heterozygosity.

	Intronic loci				Enzymatic loci								Heterozygosities	
	RP		Mlc2a		PGI-2*		PGI-3*				PGI-4*			
Alleles	100	110	100	110	080	100	090	100	110	120	080	100	Ho	He
Allelic frequencies	0.84	0.16	0.72	0.28	0.10	0.90	0.01	0.51	0.39	0.09	0.41	0.59	0.51	0.38

Discussion

Polyploidy compensates low polymorphism

Polyploidy is considered to confer more tolerance to ecological variations because the duplication of their genes provides metabolic flexibility similar to a permanent high genomic heterozygosity (Uyeno & Smith 1972, Otto & Whitton 2000, Jiang et al. 2013). The *Labeobarbus fritschi* Moroccan populations showed low polymorphism and thus low heterozygosity; however their hexaploidy may compensate this lack. Technically speaking, hexaploidy provoked a very low level of usable markers in our study due to complex multiple bands patterns at all nuclear markers.

El Gharbi (1994) analyzed a population of *L. fritschi* from the River Zerouka in her work on the phylogeny of the genus *Barbus s.l.* complex in North Africa. Her sample came from a scientific station located downstream of the Allal El Fassi dam lake analyzed in this study. It showed that the downstream population was in HWE. This author does not seem to have estimated the sex ratio of this river population.

In this 1994 study, only two allozyme loci were polymorphic (AK-2* and PGI-4*) out of the 15

analyzed in total. In this study, five loci were evidenced as polymorphic among 8 allozymic and 15 intronic interpretable markers. The polymorphic loci analyzed in the two studies were similarly scarce (respectively 2/15 and 5/23).

Sex migration dimorphism hypothesis

In agreement with the observations of Bouhbouh (2002), examination of the gonads of 39 barbel fish of 2002 from the dam lake revealed that they were all females. How can this population reproduce with only females in the lake? The two relevant hypotheses that may explain this phenomenon are: migration of male fish with seasonal meetings with females, or unisexual reproduction.

The absence of males as remarked by Bouhbouh (2002) was observed after many gillnet fishing expeditions and during all seasons between 2000 and 2001. Our own observations on a sample of 2002 are consistent with this result. If the meeting of the two sexes was seasonal, multiple fishing opportunities should have revealed some males. It is therefore very likely that no male is living in the lake. This simple observation leads us to consider unisexual reproduction as the best hypothesis.

Unisexual reproduction hypothesis

The data analysis resulted in some important features pointing in favour of the unisexual reproduction hypothesis. Deviations from HWE are important population features. Firstly, the Fis estimate showed that there was a very significant excess of heterozygotes while most panmictic deviations described in natural populations showed a deficit in heterozygotes, often due to a Walhund effect (Aurelle et al. 2002). Secondly, we found two pairs of loci (PGI-2*/Mlc2a and PGI-4*/RP) that were statistically associated ($p < 0.001$). Analysis of genotypes showed that two thirds of the specimens possessed the same genotype at the five loci studied. Given the low polymorphism at our disposal, it is questionable whether such sets of identical genotypes can be observed in fish populations that reproduce sexually. The test of clonality (Geneclone) showed that at least one multilocus genotype, repeated four times (four individuals, n°4, 14, 25 and 36, bearing the letter h in the Appendix), had a significant probability of not being propagated by sexual reproduction processes. All these results lead us to the unisexual reproduction hypothesis.

Gynogenesis

Unisexual female populations are known in fish, often after episodes of hybridization between species of the same genus (see review in Chevassus 1998). The cases of the genera *Poecilia* and *Poeciliopsis* illustrate two classical patterns of female monosex populations. *Poecilia formosa* lives in unisexual female populations, reproducing by gynogenesis (premeiotic endomitosis) with the production of diploid eggs. The sperm of another species is only used to trigger the embryonic development without participating in the genome of the future embryo as evidenced in several studies (Hubbs & Hubbs 1932, Turner et al. 1980, Castelli 1994, Ryan et al. 1996, Yang et al. 2001).

Reproduction by gynogenesis at the most maintains the initial heterozygosity (Schultz 1977, Bulger & Schultz 1979) but its reduction is expected. This mode of unisexual reproduction does not meet the Mendelian laws and causes major deviations from HWE and statistical linkages among loci.

In the hybridogenesis case of the *Poeciliopsis* complex (Moore & McKay 1971, Chevassus 1998), unisexual female populations of *P. monacha* coexist with bisexual sympatric populations (*P. lucida* and *P. occidentalis*). Thus, two forms of unisexual diploid female populations result from the hybridization between females of *P. monacha* and males of each of the two sympatric bisexual species. In these diploid females, meiosis is not recombinant but reductional and hemiclonal (hybridogenesis), allowing the genome of the mother to be transmitted without recombination: the eggs contain only the maternal genome (*P. monacha*), the paternal genome brought by the males of *P. lucida* or *P. occidentalis* participating in the somatic line only (Schultz 1980). Hemiclonal hybridogenesis maintains their hybrid status (Schultz 1977, Bulger & Schultz 1979), leading to highly polymorphic populations.

In this *Labeobarbus* monosex population, the trigging role of sympatric and even syntopic males of *Luciobarbus setivimensis* can be expected since favorable spawning areas in the dam lake are probably limited, leading to accidental gametes contacts and since *Luciobarbus* genus is a distant lineage (*Labeobarbus* is an African hexaploid lineage while *Luciobarbus* is a North African and European tetraploid one). According to the limited information produced here for the *Labeobarbus* case, gynogenesis is more probable than hybridogenesis which should follow fecondation between very different lineages (tetraploid and hexaploid of a distinct origin, Tsigenopoulos et al. 2002).

Limited nuclear genetic data is mainly due to the hexaploid nature of the *Labeobarbus* genus (Tsigenopoulos et al. 2002). Demonstrating the

mechanism of unisexual reproduction occurring in the lake requires much more information than that produced for this preliminary study. Microsatellites and introns are traditionally the main markers used in this kind of research. Despite the high number of intron system runs (15), only two proved to be interpretable for this study. It is probable that microsatellites would add a more few markers, but genome scan methods will clearly be necessary (genome scan techniques as AFLP) to disentangle the process.

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Appendix. Genotypes of the 39 barbels analyzed at five polymorphic loci. Individuals sharing the same multiloci genotypes are indicated by the same letter in the first column.

Fish numbers	enzyme PGI-2*	enzyme PGI-3*	enzyme PGI-4*	intron RP	intron Mlc2a
1	100100	100120	100080	100100	100110
6	100100	100110	100100	100110	100100
9	100100	100110	100080	100110	100100
10	100100	100100	100080	110110	100110
11	100100	100100	100080	100110	100110
15	100100	100120	100080	100110	100110
20	100080	100110	100080	100100	100100
21	100080	120120	100080	100100	100110
22	100100	100110	100080	100110	100100
28	100100	100090	100100	-	100100
31	100100	100110	100100	100110	100110
33	100100	100120	100100	100100	100110
35	100080	100110	100100	100100	100110
38	100080	100110	100080	100110	100100
a19	100080	100110	100080	100100	100110

a5	100080	100110	100080	100100	100110
b12	100080	100110	100100	100100	100100
b32	100080	100110	100100	100100	-
c24	100100	100110	080080	100100	100100
c30	100100	100110	080080	100100	100100
d17	100100	100110	080080	100100	100110
d8	100100	100110	080080	100100	100110
e23	100100	100110	100080	100100	100100
e29	100100	100110	100080	100100	100100
f13	100100	100110	100080	100100	100110
f18	100100	100110	100080	100100	100110
f2	100100	100110	100080	100100	100110
f3	100100	100110	100080	100100	100110
f34	100100	100110	100080	100100	100110
f37	100100	100110	100080	100100	100110
g16	100100	100110	100080	100110	100110
g27	100100	100110	100080	100110	100110
g39	100100	100110	100080	100110	100110
h14	100100	100110	100100	100100	100100
h25	100100	100110	100100	100100	100100
h36	100100	100110	100100	100100	100100
h4	100100	100110	100100	100100	100100
i26	100100	100120	100080	100100	100100
i7	100100	100120	100080	100100	-