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RESEARCH NOTE

Microsatellites for the gynogenetic Amazon molly, *Poecilia formosa*: useful tools for detection of mutation rate, ploidy determination and overall genetic diversity

KATHRIN P. LAMPERT[1*], DUNJA K. LAMATSCH[1,3], SUSANNE SCHORIES[1], ARMIN HOPF[1], FRANCISCO J. GARCÍA DE LEÓN[2] and MANFRED SCHARTL[1]

[1]*Department of Physiological Chemistry I, University of Wuerzburg, Biozentrum, Am Hubland, 97074 Wuerzburg, Germany*
[2]*Centro de Investigaciones Biológicas del Noreste, S.C. (CIBNOR, S.C.), Col. Playa Palo de Santa Rita, La Paz, BCS, México 23090*
[3]*Present address: Freshwater Biology, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium*

Introduction

The Amazon molly *Poecilia formosa* is a model system for studying the evolution of sex (Turner 1980; Schartl 1995) and skin cancer development (Schartl *et al*. 1997). Only very few variable microsatellites are known for this species. Microsatellites, however, have already proven to be very useful tools for diverse genetic analyses (Lampert *et al*. 2005). Here we report the results of cross-amplification of 63 microsatellite loci, originally developed for *Xiphophorus*, tested in the Amazon molly and its two parental species *P. mexicana* and *P. latipinna*. Eight microsatellite loci were found to be polymorphic. Sixteen primers allowed species identification and eight primers allowed diagnosis of different ploidy levels in *P. formosa*. These microsatellites will be useful to gain new insights into the population structures, clonal variability and reproductive biology of a mixed species reproductive complex, and also into the genetic mechanism underlying cancer formation.

P. formosa, the Amazon molly, is a small freshwater fish species that occurs in the northeastern part of Mexico and extends into the USA (Texas). It is an all-female species and was the first vertebrate discovered to reproduce clonally (Hubbs and Hubbs 1932). Its reproductive mode is gynogenesis: females produce unreduced eggs, but need sperm from a closely related sexual species to trigger the onset of oocyte development. The male's genetic material is usually excluded from the oocyte and does not contribute to the offsprings' genomes. In very rare cases, however, the exclusion mechanism fails, either leaving small parts of genetic material in the oocyte in the form of microchromosomes or leading to triploid individuals. Laboratory strains bred with Black molly can have up to three microchromosomes. A higher number of microchromosomes correlates with an increased risk of developing skin cancer (Schartl *et al*. 1997).

Amazon mollies occur in mixed schools with *P. mexicana* and with *P. latipinna*. As especially juveniles and females of *P. formosa* and *P. mexicana* are hard to distinguish morphologically we were interested in finding tools to determine the species of morphologically intermediate individuals easily. In addition, rapid determination of ploidy level is important for many questions concerning the evolutionary importance of paternal introgression. It is, therefore, also important to find genetic markers that will allow to distinguish between diploid and triploid individuals easily.

Microsatellite markers have already been shown to be of high value for studies on the clonal variability and origin of triploid clones in *P. formosa* (Lampert *et al*. 2005). Despite the considerable effort necessary to clone and isolate microsatellite markers (Hammond *et al*. 1998) they are generally considered the most powerful tools for population-genetic studies (Jarne and Lagoda 1996). A reasonable compromise to isolation and cloning is the exploitation of the relatively high conservation of microsatellite flanking regions in closely related species (e.g. Moore *et al*. 1991). Here we

*For correspondence.
Email: kathrin.lampert@biozentrum.uni-wuerzburg.de.

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report cross-amplification of 63 microsatellite loci originally developed for *Xiphophorus* (Walter *et al*. 2004).

Methods

DNA was extracted from fin-clips according to Altschmied *et al*. (1997). Primers were selected from a recently published study (Walter *et al*. 2004) according to their amplification success in *P. reticulata*. They were pretested on 16 *Poecilia* samples (2 *P. mexicana*, 2 *P. latipinna*, 7 diploid and 5 triploid *P. formosa*). PCR conditions were: 5 min of denaturing at 94°C, 30 cycles of 94°C denaturing, 55°C annealing and 72°C for extension (each step for 30 sec), followed by a final extension phase of 5 min at 72°C. In cases where no product was amplified with this protocol even an annealing-temperature gradient (52–62°C) did not uncover any products. Amplification products were analysed by electrophoresis on 5% polyacrylamide gels for 2 h at 200 V. Primers providing good and apparently variable bands were tested on the whole set of *Poecilia* DNAs (see below). Cy5 labelling was used to estimate polymorphisms and product size on an ALF sequencer (Amersham Biosciences, Freiburg, Germany). A total of 40 wild-caught *P. formosa* (25 diploids, 15 triploids) from the Rio Purificación at Barretal were used to determine clonal variation. In addition, polymorphism in 18 *P. latipinna* from several localities in Mexico (Tampico area) and the US (Florida) and 20 *P. mexicana* from several locations in Mexico (Río Purificación) were used to estimate locus variability in sexual species (for details see table 2). We used Arlequin (Schneider 2000) to determine allele frequencies and deviations from Hardy–Weinberg equilibrium.

Results

The overall cross-species amplification success was reasonably high (84%) in all species (table 1). However, of all loci that were amplified only 13% showed intraspecies

Table 1. Cross-species amplification, polymorphism tests and usefulness for scoring species and ploidy differences of 63 microsatellite markers originally developed for *Xiphophorous*.

	Amplification in			Diagnostic for	
Locus	*P. formosa*	*P. mexicana*	*P. latipinna*	Species	Ploidy levels
mCA02	m	m	m	n	n
mCA04	na	na	na	–	–
mCA05	m	m	m	n	n
mCA07	m	m	m	n	n
mCA08	m	m	m	n	n
mCA13	m	m	m	n	n
mCA16	p	p	p	n	n
mCA20	hp	m	p	n	n
mCA25	m	m	m	n	n
mCA27	m	m	m	n	n
mCA32	m	m	m	n	n
mCA41	m	p	m	n	n
mCA42	m	m	m	n	n
mCA43	m	m	m	n	n
mCA52	m	m	m	n	n
mCA62	m	m	m	n	n
mCA89	m	p	m	n	n
mCA90	m	m	m	n	n
mCA94	m	m	m	n	n
mCA95	m	m	m	n	n
mCA100	m	m	m	n	n
mCA106	m	m	m	n	n
mCA111	m	m	m	n	n
mCA113	m	m	m	n	n
mCA119	m	m	m	n	n
mCA122	m	m	m	n	n
mCA123	m	m	m	n	n
mCA130	m	m	m	n	n
mCA141	m	m	m	n	n
mCA320	m	p	m	n	n
MSC14	na	na	na	–	–
MSC49	na	na	na	–	–
MSD3	na	na	na	–	–
MSD7	m	m	m	n	n
MSD23	hp	p	p	n	n

Table 1. (*contd*)

Locus	*Amplification in*			Diagnostic for	
	P. formosa	*P. mexicana*	*P. latipinna*	Species	Ploidy levels
MSD25	m	m	m	n	n
MSD41	m	m	m	n	n
MSD65	m	m	m	n	n
mATG14	m	m	m	y	n
mATG16	m	p	m	y*	n
mATG19	na	na	na	–	n
mATG23	m	m	m	y	n
mATG31	p	p	p	y	y
mATG32	m	m	m	y	n
mATG37	m	m	m	y*	n
mATG38	p	m	m	y	y
mATG40	na	na	na	–	–
mATG41	m	p	p	y	y
mATG42	na	na	na	–	–
mATG44	p	p	p	y	y
mATG46	na	na	na	–	–
mATG61	p	p	p	y	y
mATG63	m	m	m	n	n
mATG64	na	na	na	–	–
mATG75	m	na	m	n	n
mATG76	m	m	m	n	n
mATG77	m	p	m	y	n
mATG78	p	p	p	y	y
mATG79	m	m	m	y*	n
mATG82	m	m	m	y*	n
mATG84	m	m	p	n	n
mATG86	na	na	na	–	–
mATG89	m	p	p	y	y

na, No amplification; m, monomorphic; p, polymorphic; hp, highly polymorphic (more than 15 alleles). n, No; y, yes; y*, distinguishes between *P. formosa* and each of the parental species but not between the parental species.

variability in *P. formosa* (table 1, table 2). Sixteen primers revealed products that allowed determination of the species. Eight loci could be used to determine ploidy levels of *P. formosa* individuals. Diploid individuals were heterozygous at all eight loci. Triploids showed three different alleles at six loci but only two alleles at loci mATG78 and mCA20, which could be due either to the triploids being homozygous in two of the three alleles or to null alleles. In locus mATG78 a high interspecies overlap of allele sizes was found which makes homozygosity of triploids a more likely explanation, while for mCA20 *P. mexicana* was monomorphic which might be an artefact of undetected null alleles. The number of clones detected by the different loci varied between 1 and 13 in diploids and 1 and 11 in triploids.

Variable microsatellite loci were more common in the sexual paternal species *P. mexicana* (21% of the primers tested) and *P. latipinna* (16%). In most microsatellite loci the sexual species also showed more alleles and genotypes than the asexuals (table 2). Each sexual species was monomorphic in one of the eight loci investigated in more detail (*P. mexicana*, mCA20; *P. latipinna*, mATG31). Two loci showed significant deviations from Hardy–Weinberg equilibrium for *P. mexicana* and *P. latipinna* (mATG38 and mATG78). There was no significant difference in allelic distribution between the sexes (*P. mexicana* (9 females, 10 males), $F_{ST} = 0.0059$, $P = 0.177$; *P. latipinna* (4 females, 8 males), $F_{ST} = 0.0044$, $P = 0.54$) or between sampling localities (*P. mexicana* (2 to 8 individuals per site), $F_{ST} = 0$–0.014, $P = 0.06$–0.99; *P. latipinna* (2 to 7 individuals per site), $F_{ST} = 0$–0.015, $P = 0.08$–0.99). (For more details on allele size distribution see table 2.)

Discussion

The cross-species amplification rate of primers originally developed for *Xiphophorus* was high while the number of variable loci was rather low. The microsatellite primers found, however, were of good quality. There was not much evidence for null alleles and the deviations from Hardy–Weinberg equilibrium found in the sexual species were most likely due to the wide geographic range the tested fish came from. Even though there was no geographic differentiation found this is very likely also due to low sample sizes. Allele sizes differed between sites (e.g. *P. mexicana* mATG38) and some alleles were fixed at certain sites (e.g. *P. mexicana* mATG31, MSD23) (table 2).

Table 2. Characteristics of eight microsatellite loci found to be variable in *Poecilia formosa*.

Locus	Primer sequence	*Poecilia formosa* (n = 40)[1]				*Poecilia mexicana* (n = 20)[2]			*Poecilia latipinna* (n = 18)[3]		
		Size range (bp)	No. of alleles	No. of genotypes 2n	No. of genotypes 3n	Size range (bp)	No. of alleles	No. of genot.	Size range (bp)	No. of alleles	No. of genot.
mCA16	f: CTGACTGAGATTGTGGCAGGAC r: CCTTTGAAGTTCGTAGCTGTAATGG	191–223	5	3	1	191–229	6	6	191–226	6	7
mCA20	f: GTAGCTTCTTTATGAGCAGCAGGA r: GGAGTTCTTGGCTTCGTACGA	101–225	16	5	11	101	1	1	110–190	15	13
mATG31	f: AAGCCCGAAGCTGACACTGA r: TCGCTCTGGACTGTGACCAC	74–80	3	1	1	98–116	3	4	92	1	1
mATG38	f: AGAGGGACTCTGGCAACTGACA r: CAACTCCTGGTCATCGATTCG	136–188	6	3	1	164–188	9	11	137–167	5	6
mATG44	f: CCACAATCACACCAGGGACTC r: CTGGCATCCTGTTTTCAGCTC	116–141	6	1	3	129–144	4	6	116–119	2	3
mATG61	f: GCATGATCTGAGAGGCAGAGG r: AAATAAAGGTTAGCACGACAACGTAA	132–147	6	3	1	136–148	5	9	133–145	5	9
mATG78	f: CTCTTGCTCTGATGAATACCACTCA r: CAAAACATATTCAGACACATCCAGG	192–201	3	1	1	192–210	8	10	192–207	6	7
MSD23	f: TCTGGTTGTCTTCTATTTTCTTTGAGTAA r: TGCAAACTGGAGGTCCCG	199–255	19	13	7	182–270	12	12	198–242	13	13

[1] All animals from Barretal, Tamaulipas, Mexico (N 24° 04′, W 99° 07′).

[2] Five animals from Nueva Padilla, Tamaulipas, Mexico (N 24° 04′, W 99° 07′) (3♀♀, 2♂♂), allele sizes: mCA16 191; mCA20 101; mATG31 98, 104; mATG38 164–172; mATG44 116, 119; mATG61 142, 145; mATG78, 198–210; MSD23 198; 8 animals from Altamira, Tamaulipas, Mexico (N 22° 15′, W 97° 55′) (5♀♀, 3♂♂), allele sizes: mCA16 203–229; mCA20 101; mATG31 104; mATG38 172–185; mATG44 116, 119; mATG61 136–148; mATG78 192–207; MSD23 194–242; 2 animals from Barretal, Tamaulipas, Mexico (N 24° 04′, W 99° 07′)(1♀, 1♂), allele sizes: mCA16 191–229; mCA20, 101; mATG31 98, 104; mATG38 170, 188; mATG44 116, 119; mATG61 139, 145; mATG78 192–207; MSD23 198–222; 3 animals from Rio Corona, Tamaulipas, Mexico (3♂♂), allele sizes: mCA16 191–207; mCA20 101; mATG31 98; mATG38 170–179; mATG44 116, 119; mATG61 145; mATG78, 204, 207; MSD23 238–270; in addition: one individual each from Tamasopo, San Luis Potosi, Mexico (N 21° 40′, W 99° 30′) and Mante, Tamaulipas, Mexico (N 22° 40′, W 99° 02′).

[3] Three animals from Altamira (Laguna Champayán), Tamaulipas, Mexico (N 22° 15′, W 97° 55′) (2♀♀, 1♂), allele sizes: mCA16 191–226; mCA20 175–192; mATG31 92; mATG38 137, 164; mATG44 132–144; mATG61 139–145; mATG78 201–207; MSD23 198–238; 3 animals from Altamira (Rio Puente), Tamaulipas, Mexico (N 22° 15′, W 97° 55′) (1♀, 2♂♂), allele sizes: mCA16 191–226; mCA20, 110–171; mATG31 92; mATG38 142; mATG44 129, 132; mATG61 133–139; mATG78 198, 201; MSD23 198–214; 8 animals from Flamingo Bay, Florida, USA (N 26° 25′, W 82° 01′) (2♂♂, 6 juveniles), allele sizes: mCA16 191–220; mCA20 113–190; mATG31 92; mATG38 142–167; mATG44 129, 132; mATG61, 133–145; mATG78, 195–204; MSD23, 198–238; 2 animals from San Marcos, Texas, USA (29° 55′, W 97° 55′) (1♂, 1♀), allele sizes: mCA16 191–220; mCA20 130, 190; mATG31 92; mATG38 142, 164; mATG44 132–144; mATG61 136, 142; mATG78 201, 207; MSD23 226, 242; in addition, one individual from Olmito, Texas, USA (26° 02′, W 97° 27′).

As expected the clonally reproducing *P. formosa* showed lower levels of genotypic variation than its sexual parental species (table 2). In fact, several of the primers tested that were found to be invariable in *P. formosa* were variable in *P. mexicana* (table 1) or *P. latipinna*. The primers found to be variable in *P. formosa* are, therefore, even more valuable. Our results show that they provide useful tools for future studies of clonal variability, population genetics and phylogeny in this species. In addition, a number of them can be used to easily determine the species origin of individuals and ploidy levels within *P. formosa*. The latter tasks can even be accomplished without detailed fragment size analysis simply using a 5% polyacrylamide gel with ethidium bromide staining. The noninformative primers can be used to determine mutation rates in asexual versus sexual species and, therefore, may help us understand how clonal diversity and genetic diversity are created and maintained.

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