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Microsatellite Documentation of Male-Mediated Outcrossing between Inbred Laboratory Strains of the Self-Fertilizing Mangrove Killifish (*Kryptolebias Marmoratus*)

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Abstract

Primers for 36 microsatellite loci were developed and employed to characterize genetic stocks and detect possible outcrossing between highly inbred laboratory strains of the self-fertilizing mangrove killifish, *Kryptolebias marmoratus*. From attempted crosses involving hermaphrodites from particular geographic strains and gonochoristic males from others, 2 among a total of 32 surveyed progenies (6.2%) displayed multilocus heterozygosity clearly indicative of interstrain gametic syngamy. One of these outcross hybrids was allowed to resume self-fertilization, and microsatellite assays of progeny showed that heterozygosity decreased by approximately 50% after one generation, as expected. Although populations of *K. marmoratus* consist mostly of synchronous hermaphrodites with efficient mechanisms of internal self-fertilization, these laboratory findings experimentally confirm that conspecific males can mediate occasional outcross events and that this process can release extensive genic heterozygosity.

The mangrove killifish, *Kryptolebias* (formerly *Rivulus*) *marmoratus*, is the only vertebrate species known to reproduce uniparentally by internal self-fertilization (Harrington 1961; Atz 1965; Vrijenhoek et al. 1989; Devlin and Nagahama 2002). Most individuals have a functional ovotestis that produces both eggs and sperm. Syngamy is normally internal and highly efficient (Harrington 1961, 1971), and the fertilized eggs are then deposited outside the body where they develop into hermaphroditic progeny after passing through a transitory female growth phase (Harrington 1963; Cole and Noakes 1997). Gonochoristic "primary" males are also known in nature. Although rare (<2%) at most geographic sites, they can be produced experimentally by incubating late-stage zygotes at low temperature (18–20 °C) (Harrington 1967, 1968, 1975). Secondary males (former hermaphrodites that later in life have lost their female reproductive functions) appear in laboratory strains as well (Harrington 1971; Soto et al. 1992).

Earlier molecular techniques including multilocus DNA fingerprinting have revealed extensive genetic variation in some natural populations of *K. marmoratus* (Turner et al. 1992; Lubinski et al. 1995; Weibel et al. 1999; Taylor et al. 2001), and speculation has been that such variation might be attributable to outcrossing that presumably occurs when males (who have no intromittant organ) release sperm onto occasional unfertilized eggs deposited by hermaphrodites (Lubinski et al. 1995). Here we generate primers for a large suite of microsatellite loci and then use these markers to confirm, experimentally in the laboratory, that male-mediated outcrossing can indeed occur between highly inbred strains of *K. marmoratus*.

Materials and Methods

Microsatellite Development

To enrich for microsatellites in a genomic library for *K. marmoratus*, we employed a modified version of a hybridization

capture protocol (Hamilton et al. 1999; Hauswaldt and Glenn 2003) using magnetic streptavidin beads and biotinylated probes. Briefly, genomic DNA was extracted from 25 mg of muscle tissue from an adult specimen using a DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Germantown, MD). Approximately 2 μg of genomic DNA was digested for 45 min at 37 °C with *Rsa*I or *BstU*I (New England Biolabs, Ipswich, MA). Digested fragments were ligated to double-stranded SuperSNX24 linkers (forward 5′-GTTT-AAGGCCTAGCTAGCAGAATC-3′, reverse 5′-GATTC-TGCTAGCTAGGCCTTAAACAAAA-3′) and hybridized to a cocktail of biotinylated oligonucleotide repeat probes: $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, $(ATCC)_5$, $(AC)_{13}$, $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AAC)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACCT)_6$, $(ACAG)_6$, $(ACTC)_6$, $(ACTG)_6$, $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, and $(AGAT)_8$.

The hybridization solution was mixed with magnetic streptavidin beads (Invitrogen, Carlsbad, CA), and hybridized fragments were captured on a magnetic block. Enriched DNA, recovered by precipitation, was polymerase chain reaction (PCR) amplified (25 μl total reaction volume) under the following conditions: 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.0 mM $MgCl_2$, 25.0 μg/ml bovine serum albumin, 0.2 mM each dNTP, 0.5 μM SuperSNX24 forward primer, and 0.5 units *Taq* DNA Polymerase (Promega, Madison, WI). The PCR product was ligated into a PCR 2.1-TOPO vector and transformed into One Shot Top10 Chemically Competent *Escherichia coli* cells. Positive colonies were screened for β-galactosidase activity using materials in the TOPO TA cloning kit (Invitrogen; Carlsbad, CA). Insert sizes were verified from positive colonies by PCR amplification with the M13 forward (−20) and reverse (−27) primers (0.5 μM final concentration).

Inserts ≥500 bp were purified using ExoSAP-IT (United States Biochemicals, Cleveland, OH) and sequenced with Big Dye chemistry (version 3.1, Applied Biosystems, Foster City, CA) using M13 forward or reverse primer on an ABI 3100 Genetic Analyzer equipped with 80-cm capillaries. Sequences were edited using Sequencher version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI), and primers flanking microsatellite regions were developed using OligoAnalyzer 3.0 (Integrated DNA Technologies, Coralville, IA; http://www.idtdna.com/Scitools/Applications/Primerquest/). Primers were tested by amplifying genomic DNA from adult *K. marmoratus* specimens in laboratory strains initiated many generations ago from single hermaphroditic specimens from Florida, Belize, or Honduras. PCR conditions were optimized for each primer pair, and one primer from each pair was labeled at the 5′-end in either of 2 ways: directly with a 6-FAM or HEX fluorophore (Integrated DNA Technologies) or by attaching a reverse tag (5′-GGAAACAGCTATGACCATG-3′) for tailed PCR with an M13 primer labeled with a 6-FAM, HEX, or NED (Applied Biosystems) fluorophore. The 36 sequences from which primers were developed to amplify microsatellite loci were deposited to GenBank (accession numbers DQ335412–DQ335447).

Laboratory Crosses

Crossing experiments involved highly inbred laboratory lines each propagated generation after generation by a single self-fertilizing hermaphrodite (herm) and each tracing back to a single wild herm originally collected at Utila Island, Honduras (strains Hon2 and Hon7), Belize (Bel50.91), Florida's Everglades National Park (ENP12), or Brevard County, Florida (CCHA). Each attempted cross involved placing an old (nearly senescent) herm with a secondary male in a small culture dish containing 13% saltwater solution (Harrington 1971). To prevent a fish from eating its own eggs and to provide shelter, needlepoint mesh (4.0 mm) and anchoring pebbles were distributed around the bottom of the dish. Eggs were harvested 96 h after deposition and incubated in a covered glass culture dish at 27–28 °C. On hatching, fry were fed brine shrimp until they reached 10-mm standard length, at which time they were transferred to covered plastic culture containers and fed Tetramin flakes until euthanized for DNA extraction.

DNA Extractions

High molecular weight genomic DNA samples were isolated using a standard guanidinium isothiocyanate (GIT) chaotropic salt procedure (Turner et al. 1989). Approximately 0.5 gm of muscle tissue was excised from each specimen, minced on a cold glass plate, and gently homogenized in a 4 M GIT solution. DNA was next extracted using phenol/chloroform followed by pure chloroform/isoamyl alcohol and then precipitated in cold 95% ethanol in the presence of ammonium acetate salt. The resulting nucleic acid pellets were air-dried and redissolved in 1.5 ml distilled, deionized water.

Genotypic Assays

Amplifications of microsatellite loci with fluorescently labeled PCR primers were performed in a 12.5-μl reaction volume containing 1 μl of purified genomic DNA, PCR Master Mix (Promega), and 0.5 μM of both forward and reverse primers. Tailed PCRs were performed in a 10-μl volume containing 1 μl of purified genomic DNA, 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 25.0 μg/ml bovine serum albumin, 0.2 mM each dNTP, 0.25 μM M13-labeled primer, 0.25 μM locus-specific primer, 0.025 μM tailed locus-specific primer, and 0.4 units *Taq* DNA Polymerase (Promega). PCR products (1.5 μl) were mixed with 2.5 μl deionized formamide, 0.5 μl of either GeneScan-400HD[ROX] or GeneScan-500[ROX] internal lane standard, and 0.5 μl loading dye. Samples were denatured in a 95 °C heating block for 3–5 min and chilled on ice before being loaded onto 5% acrylamide gels. Samples were electrophoresed on an ABI 377 DNA Sequencer at 3000 V for 2 h at 55 °C. Alleles were sized using the software packages GeneScan version 3.1.2 and Genotyper version 2.5 (Applied Biosystems).

Table 1. Features of 36 microsatellite loci isolated from 7 *Kryptolebias marmoratus* inbred strains

Locus	Repeat motif	Primer sequence 5′–3′	T_a[a]	No. alleles observed (size range, bp)	H_e[b]
B10	$(CA)_{21}$	[c]F: TCTGCCCAGTGGTTTATGA R: TTACCACCTGCCACGTTATTGC	53	3 (131–137)	0.57
B86	$(CTT)_4N_n(GAT)_7$	F: ACATGGCTAGCTTTGCTGATGC [c]R: TGAAAGTTTCGTCCCGCTGGTT	53	1 (189)	0
R1	$(AATG)_{12}$	F: 6Fam-ACAACGAAATAAAACAGTATCTGA R: CTTGCTTTGATAGTCTAATGAAGA	55	2 (246–250)	0.23
R3	$(ACAG)_{24}$	F: 6Fam-AATTTTATGTATCTGGACACAGG R: TAATACACTTCTACAGCCAAGGT	55	4 (126–186)	0.70
R4	$(CCTGT)_{18}$	F: 6Fam-CACTTTCCAATCACCTCCT R: CAGCACAAGAAAACGAAAA	55	3 (225–245)	0.43
R5	$(AATC)_{15}$	F: Hex-CATCATCACTGTCACCATATTT R: TGGACCTATTTGTGTGTCTTTA	55	5 (290–310)	0.83
R6	$(AG)_8(ACAG)_{12}$	F: GCTTCGCTTGGTGAAAC R: Hex-AACACTCCGGTATGCGT	50	3 (198–214)	0.70
R7	$(ATCC)_{10}$	F: 6Fam-CCTTAGTTCTAACAACACCCAT R: TGGTCTCTTGTGTTTGGTATTA	55	1 (188)	0
R9	$(AATC)_{13}$	F: 6Fam-TCCTTCCTGGTTCCTTTC R: GCTACCCCTGTGGTTCTT	55	4 (208–228)	0.70
R10	$(AAG)_{27}$	F: GAAACATGTCCTCATACTCCA R: Hex-TAAACCTCTGTTATCTGCTGC	55	6 (210–240)	0.87
R11	$(AAAG)_4AAGG(AAAG)_{16}$	F: 6Fam-CTGCACTAAGTGGATCTGTTC R: TTGTTACACCAATCATTACCC	55	4 (166–186)	0.77
R16	$(ATTG)_{11}$	F: 6Fam-AGCACTTTGGTTACCCCTA R: GAGCATCAAATAAGCAGCA	55	4 (284–300)	0.70
R17	$(ACAG)_{10}(AAAG)_{11}$	F: GCCTCACTGCTGCTACTACT R: Hex-AACTCAACAACGAACGAAAT	55	5 (258–298)	0.80
R18	$(AATC)_{17}$	F: 6Fam-CTGTGTGTTGAACTGACCTG R: CCACTTATTAGTGACTCGGC	55	4 (206–262)	0.73
R19	$(AAAG)_{14}$	F: 6Fam-ACACACAGTCAAGCACAAGA R: AATTAGGTAGAGACCCAGCC	55	4 (120–144)	0.73
R22	$(ACAG)_{10}$	F: 6Fam-CTCGCTGCTACTATTGCTG R: CCGTATGGGTTGTTCTTTT	55	5 (166–202)	0.83
R23	$(ATTG)_{17}$	F: 6Fam-TTAACAAGCCTCTCCTGTGT R: CATATGTCGCACAGATTTCA	55	4 (256–272)	0.77
R24	$(ATTG)_{13}$	F: GCTACCCCTGTGGTTCTT R: Hex-TCCTTCCTGGTTCCTTTC	55	4 (208–228)	0.70
R25	$(AAAC)_{18}$	F: 6Fam-TGGCTAAACAAACAAACAGA R: CATGAGTAGGGTTGTCCTGT	55	5 (100–132)	0.80
R26	$(ACAG)_{16}$	F: 6Fam-CAATTTGTGCTTAAACTTGCT R: ACAATCACCAAGAAATCAAGA	55	3 (194–202)	0.67
R27	$(AATC)_{24}$	F: 6Fam-TTTCATGTTCTGTATCTTTGTTTT R: ACTTTTACTTTTGCCATTTACTCT	55	6 (154–198)	0.83
R28	$(ACTC)_{12}$	F: 6Fam-ACACAGACAGACAAACGGA R: TGGCCAAACAATTAAACAA	55	2 (174–178)	0.53
R30	$(AATC)_{14}$	F: 6Fam-CTTATAGGGTGGGACAGAATTA R: CTTAAGAGGCAAGAGCAGTTAT	55	3 (160–168)	0.67
R33	$(ACAG)_{20}$	F: 6Fam-CGTGTATGTGTGCAGAGC R: GAGAGAGAGGAAGGCAGC	55	2 (188–220)	0.50
R34	$(ACAG)_{17}$	F: 6Fam-TACCAGATCTTTGAGACAGACA R: ATCTCTGACAAAATCCTTCAAC	55	2 (148–204)	0.50
R35	$(AC)_{14}$	F: CTTCTTTCAGCTTCTTGTTTTT R: Hex-CTGCATACTCTGACACTGATGT	55	3 (140–156)	0.57
R36	$(ACAG)_{14}$	F: 6Fam-TAAACAGCTGCACGGTTAG R: GGAGGTCGATGTTTTCTGT	55	1 (182)	0
R37	$(ATCT)_{44}(GTCT)_{11}$	F: 6Fam-CTGATCCCTGAACTAAATCCTA R: GCATTCATTGATGTTCTACTTG	55	7 (268–340)	0.90
R38	$(AAAG)_3AGAG(AAAG)_{16}$	F: 6Fam-TGCCTCCAAACCAGTCTA R: CCACCAATGGACTGAGAA	55	4 (190–210)	0.77
R45	$(CCAT)_{10}$	[c]F: CTTCTGGTTTGGCTGGTCAATAGC R: AGCACTTTGGCCAATGGCAGAT	53	1 (254)	0

Table 1. Continued

Locus	Repeat motif	Primer sequence 5′–3′	T_a[a]	No. alleles observed (size range, bp)	H_e[b]
R86	$(GT)_{21}$	[c]F: AAGGGTCAGGGTTAGGCTGTAGTT R: AACCACACAACACATGCAGGGA	53	4 (182–218)	0.77
R90	$(GT)_{28}$	[c]F: TCTCTCGCTGCATGTCAATGCT R: AGAGCTGGCTTGCTCTCTTGAT	53	4 (226–254)	0.73
R92	$(GT)_3AT(GT)_{15}$	F: TAAGCTCAAACATGGCACCGCT [c]R: ATGATGACTCCACACACCCTCA	53	2 (198–204)	0.23
R93	$(GATG)_{20}$	F: ATATCCTGCCCTTGTTGCCTCT [c]R: TGCTGCCTCAGACACAGCTC	53	4 (156–168)	0.77
R103	$(CA)_{18}$	F: TTCTCTGCACTCAATGGGCTGT [c]R: ACTCACGTTCCCACTGGTTGT	53	2 (140–144)	0.40
R112	$(AC)_{21}$	[c]F: AGACGGCCATATGCTCCAATCA R: TCCGAACTAAAGAGGCACCCAA	53	5 (201–221)	0.73

[a] T_a, annealing temperature.

[b] H_e, unbiased expected heterozygosity (Lewis and Zaykin 2001).

[c] Primer contains M13 reverse tail at 5′-end; an M13 reverse oligo labeled with 6-FAM, HEX, or NED was used in PCR.

Results

Microsatellite Markers

Table 1 describes repeat motifs and primer sequences for the 36 microsatellite loci developed and employed in the current study. Four of these loci (B86, R11, R38, and R92) contained interrupted repeat motifs, 3 (R6, R17, and R37) contained compound repeats, and 29 were composed of pure repeats as follows: dinucleotide motifs, 6 loci; trinucleotide motif, 1 locus; tetranucleotide motifs, 21 loci; and pentanucleotide motif, 1 locus. Among the pure dinucleotide microsatellites, the AC/CA motif was most prevalent (4 of 6 loci). Among the pure tetranucleotide markers, 9 unique repeat types were observed of which ACAG was the most represented (6 of 21 loci).

Based on 42 specimens from the 7 laboratory strains, 32 of the 36 microsatellite loci (89%) were polymorphic, and observed numbers of alleles per locus ranged from 1 (loci B86, R7, R36, and R45) to 7 (locus R37) with a mean of 3.5 (Table 1). Heterozygosities (expected Hardy–Weinberg values for an artificially pooled sample involving one individual per strain) ranged from 0 to 0.90 across loci (Table 1), with a mean of 0.59. There was no appreciable difference between dinucleotide-repeat loci and tetranucleotide-repeat loci in terms of mean numbers of alleles per locus (3.5 vs. 3.3) or in the mean expected per-locus heterozygosities (0.62 vs. 0.60).

This extensive genetic variation was distributed almost entirely among (rather than within) strains because, as expected, inbred lines were highly homozygous. Indeed, with a single exception (locus 37 in one specimen from the Hon2 strain), no individual was observed to be heterozygous at any surveyed locus, and different assayed specimens from the same strain were genetically identical across all 36 loci. By contrast, individuals from different strains were genetically well differentiated showing fixed allelic differences from one another at a minimum of 15 loci (strains Ver5 vs. CCHA and Hon2 vs. Hon11) and at a maximum of 29 loci (strain ENP12 vs. both Hon2 and Hon7). On average, pairs of laboratory strains differed at 24 of the 36 microsatellite loci (66%), so these markers offer great power to detect outcross events.

Laboratory Crosses

Genetic evidence on selfing versus outcrossing was unambiguous, as exemplified by results of the crossing experiment involving a Hon2 herm and an ENP12 male. Five of six juveniles from this attempted cross were identically homozygous at all 36 loci for alleles also displayed in homozygous form by what therefore must have been their sole Hon2 parent. The remaining juvenile (#6) was heterozygous at all 29 diagnostic loci for alleles that distinguished its Hon2 dam from its ENP12 sire, so it clearly arose from an outcross event. By similar lines of evidence, 19 juveniles from the attempted cross between a Hon7 herm and an ENP12 male proved to be products of self-fertilization by their Hon7 parent, but one other juvenile in that experiment reflected an outcross fertilization. Finally, in the attempted cross between a Bel50.91 hermaphrodite and CCHA male, all 6 juveniles surveyed proved to be self-fertilization progeny of the Bel50.91 herm. Thus, overall for the 3 attempted interstrain crosses, 30 of 32 surveyed progenies (93.8%) were products of self-fertilization by a hermaphrodite, but the other 2 individuals (6.2%) clearly resulted from outcrossing between a hermaphrodite and a male.

#26

#26

One specimen that proved to be an outcross hybrid (individual #6 from the Hon2 × ENP12 cross) had fortuitously been kept alive for production of F2 progeny. It produced 6 F2 offspring that we then genotyped at the 29 loci known to be heterozygous in that specimen. As shown in Table 2, neither did the loss of multilocus heterozygosity in 5 of these 6 F2s depart significantly from the neutral expectation of 50% decline after one generation nor was the overall trend significant after applying the Dunn–Šidák correction (Sokal and Rohlf 1995) for multiple progeny comparisons. Also, the

Table 2. Heterozygosity remaining in 6 selfing-derived F2 progenies of an F1 outcross hybrid (between parental strains Hon2 and ENP12) that was heterozygous at 29 microsatellite loci

F2 individual	No. heterozygous loci remaining (among 29 possible)	Heterozygosity lost (%)	χ^2	*P* value
a	19	34.5	2.79	0.095
b	9	69.0	5.83	0.041[a]
c	13	55.2	0.31	0.58
d	15	48.3	0.03	0.85
e[b]	14	51.7	0.04	0.85
f	15	48.3	0.03	0.85
Pooled	85	50.6	0.21	0.88

[a] Nonsignificant after correction for multiple comparisons (Dunn–Šidák test).

[b] This individual was screened at only 27 loci.

grand pooled loss of heterozygosity (50.6%) did not depart significantly from the 50% null expectation under random Mendelian segregation.

If the marker loci are not tightly linked, then the expected per-locus number of heterozygous individuals (which can vary from 0 to 6 in this collection of F2 progeny) should follow a binomial distribution centered in this case on 3.0 (Sokal and Rohlf 1995). The empirical distribution of heterozygous individuals (Table 3) did not differ significantly from this null expectation ($\chi^2 = 10.01$, degree of freedom [df] = 6, $P = 0.12$), so we cannot refute the working hypothesis that most of the loci described here segregate and assort independently in simple Mendelian fashion.

Discussion

Earlier lines of evidence for male-mediated outcrossing in *K. marmoratus* were either indirect or artificial. Based on high DNA fingerprint diversity observed in a natural population from Twin Cays, Belize, Lubinski et al. (1995) posited that males known to be present in that population might sometimes fertilize virgin ova deposited externally by hermaphrodites, thereby generating recombinant population genetic variety beyond what de novo mutations alone could produce (see Mackiewicz et al. 2006a for more recent microsatellite evidence on this likelihood). Earlier, Harrington (1967) and Harrington and Kallman (1968) had attempted forced crosses between a laboratory-generated male from one highly inbred strain and a male-sterile hermaphrodite, that oviposited unfertilized eggs, from another, and they showed that hybrid progeny could be produced by this procedure. These researchers also showed that F1 progenies were able to accept tissue grafts from both parents but that parents rejected transplants from an F1 donor, suggesting (indirectly) that F1 progenies were heterozygous and the parents were homozygous at relevant histocompatibility loci. Our current genetic data go beyond those of previous reports by directly demonstrating that *K. marmoratus* males can indeed outcross successfully with functional hermaphrodites and that the process can generate highly heterozygous and fertile offspring.

None of the available evidence for *K. marmoratus* eliminates the possibility that outcross events might also occur occasionally between functional hermaphrodites. Regardless of whether this latter possibility is ever realized, our data confirm that male-mediated outcrossing can take place and can be a powerful force in generating multilocus heterozygosity that subsequently becomes available (on a resumption of normal self-fertilization) for rapid conversion into new recombinant inbred strains.

Previous genetic studies of *K. marmoratus* have rested primarily on traditional multilocus DNA fingerprinting, but the molecular probes involved in such assays are notoriously ill-suited for revealing genotypes at particular loci or for quantitatively assessing the important within-specimen (i.e., heterozygosity) component of population variation. As we demonstrate elsewhere (Mackiewicz et al. 2006a, 2006b), the large bank of microsatellite markers that we have developed here afford novel opportunities to analyze the mating system and population genetic patterns of *K. marmoratus* in nature as well.

Table 3. Distribution of numbers of individuals heterozygous per locus in 6 selfing-derived F2 progenies of the F1 hybrid between strains Hon2 and ENP12

Genetic category (possible per-locus number of heterozygotes among 6 specimens surveyed)	Expected number of loci in this genetic category[a]	Observed number of loci in this genetic category[b]
0	0.4	1
1	2.5	1
2	6.3	11
3	8.4	3
4	6.3	8
5	2.5	2
6	0.4	1

[a] Binomial expectation assuming independent assortment across loci.

[b] Departure from the expected distribution is nonsignificant ($\chi^2 = 10.01$, df = 6, $P = 0.12$).

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