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Genetic Monogamy in the Channel Catfish, *Ictalurus punctatus*, a Species with Uniparental Nest Guarding

ANDREY TATARENKOV, FELIPE BARRETO, DANA L. WINKELMAN, AND JOHN C. AVISE

Behavioral observations have suggested that Channel Catfish, *Ictalurus punctatus*, spawn as monogamous pairs and that males alone provide subsequent care to the resulting embryos and fry. However, genetic monogamy is quite uncommon in fish and is not necessarily correctly predicted by apparent social interactions. Here we develop and employ seven microsatellite loci to address biological parentage and the genetic mating system in a natural population of *I. punctatus*. A total of 175 progeny and their respective attendant males were genotyped from five nests. Results indicate that each male had mated with only one female in his nest. Additionally, one nest contained a second group of full sibs unrelated to the attendant male and his mate who proved to be the biological parents of all other progeny within that nest. This instance probably represents either a case of nest piracy by the attendant male or perhaps our inadvertent sampling of progeny from two closely adjacent nests. In any event, our findings help confirm a rare suspected example of genetic monogamy in a fish species with uniparental offspring care.

THE Channel Catfish (*Ictalurus punctatus*) accounts for 43% of all aquaculture production in North America (FAO, 1997) and ranks among the ten most widely consumed aquaculture species worldwide (Naylor et al., 2000). It is also a popular game fish in the wild (Wydosky and Whitney, 2003). Native to North America, *I. punctatus* was introduced to Europe in the 1930s and since has expanded its range to many countries. Despite the species' economic importance, some aspects of its biology (e.g., natural population genetic structure and mating behavior) remain poorly understood, probably in part because spawning is secretive in nature (Sigler and Sigler, 1987) and population densities are often low. Previous studies employing molecular genetic markers have been confined primarily to investigations of polymorphism levels and genetic divergence patterns in domestic or feral stocks (e.g., Hallerman et al., 1986; Waldbieser et al., 2001; Mickett et al., 2003).

Ictalurus punctatus spawn during the spring and early summer (Becker, 1983; Sigler and Sigler, 1987). Males excavate nests under overhangs or logs (Wydoski and Whitney, 2003), and the female (unlike in some other congeneric species) does not assist in nest construction (Smith, 1979). What little is known about spawning behavior itself has come mostly from aquarium observations (Clemens and Sneed, 1957). After a period of biting and nudging, the pair positions itself anti-parallel (facing in opposite directions), occasionally wrapping a tail around the head of the partner. The process can last 4–6 hours and results in the deposition of approximately 1,600 to 70,000 eggs that may be externally fertilized by the male's release of milt (Wydoski and Whitney, 2003).

After spawning, the male drives the female away and then alone guards and tends the eggs, remaining at the nest throughout the incubation period of five to ten days (Wydoski and Whitney, 2003). The male cleans and aerates the fertilized eggs by mouthing and fanning. After hatching, fry normally remain for about a week within the nest where they continue to be defended by the attendant male (Becker, 1983). A male may spawn more than once per season but a female reportedly spawns only once a year (Becker, 1983; Sigler and Sigler, 1987).

In the absence of direct genetic evidence, either of two diametrically opposing hypotheses (or any mixture of the two) might justifiably be advanced regarding biological parentage and familial composition in *I. punctatus* broods. An adult male's tenacious protection of eggs and fry, coupled with his large body size (at least 25–30 cm; Becker, 1983), suggests that nest defense against potential cuckolders could be effective such that all progeny within a typical nest may have the same sire; on the other hand, multiple paternity is known from molecular markers to be common in many other nest-tending fish species (review in Avise et al., 2002). Also, aquarium observations suggest that spawning in *I. punctatus* may normally involve monogamous pairs such that a typical nest would contain full-sib progeny only; but on the other hand, nest-tending males in many other fish species are known to spawn with up to several females simultaneously or in quick succession such that a typical nest contains

mixtures of full-sib and half-sib cohorts (Avise et al., 2002).

Microsatellites have proven to be exceptionally powerful genetic markers for elucidating genetic paternity, maternity, and mating systems in numerous organisms. Here we develop and apply seven microsatellite loci to questions about biological parentage and sibship composition in natural *I. punctatus* nests from the wild.

Materials and Methods

Animal collection.—All *I. punctatus* specimens were collected from Lake Carl Blackwell, Oklahoma. Adult attendant males and eggs containing unhatched fry were collected by handfishing or ''noodling'' from five nests (designated A–E, below). Noodling is a fishing technique in which a fisherman walks or swims in shallow water, searching under structures such as rocks, ledges, and banks for nest-tending catfish. Fish are then ''grabbed,'' usually by the lower jaw or opercular opening, pulled from the nest, and placed on a stringer. For the current study, the collector returned to the nest and retrieved (by feel of hand) the egg mass. A small subsample of eggs was taken in the field by removing eggs haphazardly from several places in the egg mass and these were preserved in buffer. We did not collect the entire egg mass due to the logistical constraints. Tissue samples were also taken from the adult males by removing a small amount of muscle directly below the adipose fin and preserving it in ethanol. Afterwards fish were released alive. The number of fertilized eggs (progeny) taken from each nest was as follows: nest A, approximately 150; nest B, 20; nest C, 23; nest D, 46; and nest E, 39. From nests B–E, all collected progeny were genotyped; from nest A, 47 offspring were included in the microsatellite analyses.

Laboratory procedures.—Genomic DNA was extracted from whole eggs and fry, or from 50–100 mg of adult tissue, using proteinase K tissue digestion followed by phenol-chloroform-isoamyl extraction and ethanol precipitation (Milligan, 1998). We used a modification of the protocol by Hamilton et al. (1999) to isolate microsatellites (Hauswaldt and Glenn, 2003). Four μg of genomic DNA (from the adult male in nest A) was digested with restriction enzymes that form blunt ends (e.g., BstUI and Rsa I [New England Biolabs]). Digested fragments were ligated to double-stranded SuperSNX24 linkers (forward 5′-GTTTAGGCCTAGCTAGCAGAATC-3′; reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-3′) to provide PCR priming sites. The DNA fragments with linkers were then hybridized to a mixture of biotinylated oligonucleotide probes (motifs such as $(AG)_{12}$, $(AC)_{12}$, etc.), captured on streptavidin-coated magnetic beads (Dynal), washed and recovered from magnetic beads by heating, and ethanol precipitated.

The fragments enriched for microsatellite repeats were then subjected to an additional enrichment step of hybridization, bead capture, wash, and recovery (as described above). The recovered DNA fragments were next amplified via PCR using linkers as priming sites and cloned using the TOPO TA cloning kit (Invitrogen). Transformed bacteria were grown on LB plates with X-gal (Sigma), and positive colonies were selected for amplification by PCR with M13 Forward (−20) and M13 Reverse (−29) primers (Integrated DNA Technologies). PCR products were run on 1% agarose gels to determine sizes of the DNA fragments. PCR products containing fragments of proper size (500–1000 bp) were purified using ExoSAP-IT (USB) and prepared for sequencing using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ver 3.1, Applied Biosystems). Sequencing was conducted on an ABI 3100 Genetic Analyzer equipped with 80-cm capillaries. Primers were designed based on obtained sequences using the program PrimerQuest (Integrated DNA Technologies). After primer testing and PCR optimization using the genomic DNA from five males, seven polymorphic loci were selected to conduct the parentage study.

For genetic screening at the population level, we used tailed PCR to produce fluorescently labelled DNA fragments (Oetting et al., 1995). The 5′-end of one primer in each pair had the attached tail, consisting of 19 bp of the M13 reverse primer 5′-GGAAACAGCTATGACCATG-3′. An M13 reverse primer, fluorescently labelled with one of 6-FAM, HEX, or NED fluorophores, was included in the PCR reaction, resulting in a labelled product detectable on an ABI 377 automated sequencer. PCR amplifications were conducted in 10 μl of the following mix: 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.025 μg/μl bovine serum albumin, 0.2 mM of each dNTP, 0.25 μM M13 reverse primer, 0.25 μM of locus-specific primer without tail, 0.025 μM of locus-specific primer with tail, 0.4 units Taq DNA Polymerase (Promega), and approximately 10–40 ng of genomic DNA.

All loci were amplified separately under the following conditions: initial denaturation step at 95 C for 5 minutes, followed by 34 cycles of 95 C for 40 sec, annealing temperature (see Table 1) for 40 sec, 72 C for one minute, and a final extension step at 72 C for seven minutes. Prior to

TABLE 1. CHARACTERIZATION OF SEVEN MICROSATELLITE LOCI NEWLY ISOLATED FROM THE CHANNEL CATFISH, *Ictalurus punctatus*. T_a, annealing temperature; N_a, number of alleles observed (based in effect on 12 adult specimens); H_e, expected heterozygosity (gene diversity).

Locus	Repeat motif	Primer sequences	T_a	Observed step size	N_a (size range)	H_E
BM1-3	$(GAT)_7$	*F: *Ned*-TACTCAGAAATCTCAGCCCGGT	52	3	6 (165–180)	0.841
		R: AGGACCCAGGATCCAGCAAGA				
BM1-31	$(GT)_{10}(AGT)_{10}$	F: CACCGGCTTGTATTTGACCTGT	51	3	7 (247–289)	0.765
		*R: *Hex*-AAATGCTTGCAGAACGGGA				
BM1-33	$(GT)_{15}$	F: TCAGGTTCGTCTTCAGACTTGAG	51	2	9 (257–283)	0.913
		*R: *Fam*-AGTGCAGCTCCAGTCAACATCA				
BM1-37	$(GT)_{20}$	F: GTCCGGACATGCCTACAGAATA	51	2	9 (154–186)	0.909
		*R: *Hex*-CATTTCACAGCAACCTCCC				
BM1-48	$(CT)_{25}$	F: TGCATACGCACTCCTACACGTCAA	52	2	15 (182–230)	0.942
		*R: *Fam*-TTAGCCTCGCTAATGAGCTGGA				
BM2-24	$(GTT)_8N(CT)_{10}(AC)_{14}$	*F: *Ned*-ACGACGGTTCATCATTGCCGTA	51	2	6 (282–296)	0.739
		R: AGGTAGGAGATGCTTTCGGTCT				
BM2-32	$(AATG)_8$	*F: *Ned*-ACGACTGGACATGTGAAGACCA	51	4	4 (238–250)	0.489
		R: TAACAGAACACCTGCAGTGGGA				
Average					8	0.785

* The complete sequence of the primer includes *M13 Reverse* tail (5′-GGAAACAGCTATGACCATG-3′) at its 5′-end. *M13 Reverse* oligo labelled with 6-Fam, Hex, or Ned was used in the PCR reactions.

electrophoresis, PCR products of seven loci were pooled in equal proportions, and 1 µl of the poolplex was combined with 0.4 µl of GeneScan-500[Rox], 0.8 µl of loading dye (both from Applied Biosystems), and 2 µl of deionized formamide. Samples were denatured in a 95 C heating block for three minutes and chilled on ice before being loaded onto 5% acrylamide gels. Samples were electrophoresed on an ABI 377 DNA Sequencer at 3000 V for two hours at 55 C. Alleles were sized using the software packages GeneScan version 3.1.2 and Genotyper version 2.5 (Applied Biosystems).

Genetic and statistical assessments of nests.—When alleles in the multi-locus genotype of an attendant male and his suspected progeny were consistent, maternal multi-locus haplotypes in each offspring could readily be deduced by subtraction (e.g., DeWoody et al., 2000). When the alleles in some progeny did not match those of their attendant male, multi-locus genotypes of sires and dams could not be determined reliably, but it usually did remain possible to deduce parental genotypes separately for each locus.

Observed genotypic proportions in progeny cohorts were tested for conformance to the expected Mendelian ratios using χ^2 statistics (Zar, 1984). Two offspring with apparent *de novo* mutations (see below) were excluded from these tests. From these tests of significance, probabilities were combined using Fisher's approach as detailed by Sokal and Rohlf (1995:794–797). Corrections for multiple tests were performed using Dunn-Šidák's multiplicative inequality for calculating critical values of the chi-square distribution (Sokal and Rohlf, 1995:239–242). Unbiased expected heterozygosities were calculated using the program GDA (Genetic Data Analysis: Computer Program for the Analysis of Allelic Data. Version 1.0 (d16c). P. O. Lewis and D. Zaykin, 2001. Free program available at http://lewis.eeb.uconn.edu/lewishome/software.html). Probabilities of parentage exclusion were calculated according to Jamieson and Taylor (1997).

RESULTS

Genotypes for seven microsatellite loci were determined for all five attendant males and a total of 175 of their putative progeny. In four of these nests (A, B, C, and E), at each locus one allele in each offspring matched an allele in the respective nest-attendant male (for a single exception see the *New mutations* section below). Thus, for each of these progeny, it was straightforward (by subtraction) to specify the alleles as well as the precise gametic haplotype of maternal origin. Overall, the resulting genotypic patterns for the dams indicated that for each nest, only one female had contributed genes to the entire cohort of assayed progeny.

In nest D, however, several progeny displayed genotypes that could not have been derived from the resident male. To determine the number of parents in this case, the following steps were conducted. First, we separated specimens evi-

dently fathered by the resident male (24 individuals) from the remaining progeny (22 individuals). Second, we determined by subtraction that a single female could account for all genotypes recorded in the progeny of the resident male. Next, we observed that the remaining progeny (those not fathered by the resident male) collectively carried at most four alleles (range 2–4) at any locus, hinting that only one pair of parents was involved. Although it was not possible to assign particular genotypes to the male or female, from basic rules of the Mendelian inheritance we could reasonably conclude that a genetic contribution from one male and one female was sufficient to explain the genotypic composition of this portion of the brood. Furthermore, it was obvious that these genotypes did not originate from either the resident male or his deduced first mate (i.e., the dam of the other half of the brood). Thus, nest D clearly contained two independent sets of progenies from two separate pairs of parents.

New mutations.—Among all progeny examined, we found two individuals that were inconsistent at one locus (and one locus only) with one of their provisional parents. At face value, offspring B16 from nest B lacked an allele from its putative mother at locus BM1-3, and offspring E40 from nest E lacked an allele from its putative father again at locus BM1-3. Otherwise, at all other surveyed loci both of these progeny carried alleles that matched the states of those in their respective provisional parents.

The expected frequencies of such six-locus matches in the population at large were very low in both cases (probabilities of identity were 1.25×10^{-8} and 1.2×10^{-8}, respectively). Thus, we conclude that no additional parents had been involved for individuals B16 and E40, but instead that a total of two *de novo* mutations had arisen. In individual B16, this mutation was of maternal germ-line origin and involved either a conversion of the dam's BM1-3^{174} or BM1-3^{177} to allele BM1-3^{168} (by a loss of two or three repeats), or, alternatively, by a mutation in a priming region of one of those alleles resulting in a null allele (such that only the paternal BM1-3^{168} was detectable). In individual E40, the new mutation apparently arose in the paternal germ-line and involved a conversion of the dam's BM1-3^{171} or BM1-3^{174} allele to allele BM1-3^{168} (by a loss of one or two repeats).

Genotypic fit to Mendelian ratios.—As described in the initial analyses above, the genetic data were consistent with the presence of progenies derived from a total of six pairs of parents in the five nests. In each of these six full-sib progeny cohorts, all single-locus genotypes expected from the deduced genotypes of the respective parents were present. However, the parents of nest E must have been identically homozygous at locus BM2-32, and similarly the resident male from nest D was identical to the dam at locus BM2-24. Thus, allelic ratios at these loci could not be tested for conformation to Mendelian inheritance in these particular crosses. Of the remaining 40 combinations that could be statistically treated (seven loci × six crosses, minus two), χ^2-tests detected significant departures of observed from expected genotypic ratios in only two cases (locus BM1-3 in family A and locus BM1-37 in family D), and even this low level of significance was lost after Dunn-Šidák's correction for multiple tests. There was also no tendency for departures from Mendelian ratios either at a particular locus across all families or in a particular family across all loci, as judged by the Fisher's combined probability tests.

Level of variation.—Although our estimates of population variability are based on only 12 adult individuals, we nonetheless observed an average of eight alleles (range 4–15) per locus. Expected single-locus heterozygosities ranged from 0.49 to 0.94, with a mean of 0.78. Observed and expected heterozygosities at each locus invariably fit Hardy-Weinberg equilibrium.

Discussion

Genetic variability and exclusion probabilities.—Levels of genetic variation detected in this report (mean heterozygosity = 0.78, mean number alleles per locus = 8) are similar to the mean heterozygosity (0.70) and mean number alleles per locus (8) reported earlier for a different suite of microsatellite loci in another outbred population of *I. punctatus* (Waldbieser and Bosworth, 1997). Such extensive microsatellite variation has previously been put to service in developing an extensive linkage map for *I. punctatus* (Waldbieser et al., 2001) that in turn may assist in breeding programs for the genetic improvement of stocks in this species. Here we have employed microsatellites for an entirely different purpose–to assess genetic parentage and mating systems in the wild.

The seven microsatellite loci surveyed here provided outstanding power for resolving parent-offspring associations. Thus, the parent-pair exclusion probability was 0.9999, the probability of maternity exclusion (when the genotype of the father is known) was 0.9992, and the probability of single-parent exclusion (when no information

on the other parent is available) was 0.9877. Further indication that our set of loci was powerful for discerning parent-offspring links was the finding that no two individuals (not even in the same family) were identical across all seven loci.

A high mutation rate at microsatellite loci undoubtedly helps to account for the high heterozygosities observed in this and other such studies. Indeed, in the current case we documented two *de novo* mutations (both at the same locus, one being in the paternal and the other in the maternal germ line). Because we screened in effect a total of 2450 gametes [175 diploid progeny × 7 loci], this yields an estimated mutation rate of 8.1×10^{-4}, which is well within the range of reports for other species (Goldstein and Schlötterer, 1999).

Genetic mating system.—We found no evidence for departures from genetic monogamy in the *I. punctatus* nests examined. In other words, all five nests sampled contained full-sib cohorts exclusively. Actually, one apparent nest (D) contained two distinct full-sib cohorts (i.e., from two separate pairs of parents), a result that likely indicates one or the other of the following: perhaps two adjacent nests were inadvertently sampled; or, perhaps the attendant male had pirated another male's nest already containing fertilized eggs, and then himself spawned with a new female. In any event, the fact remains that we detected no half-sib assemblages that would indicate multiple mating in a given nest by either a particular male or a particular female parent.

The presence of full-sibships within an *I. punctatus* nest does not by itself prove genetic monogamy because it cannot exclude the possibility that a female lays her eggs in more than one nest. However, aquarium observations suggest that a female typically deposits all of her ripe eggs during a spawning event with one male (Clemens and Sneed, 1957) and that she spawns only once per breeding season (Sigler and Sigler, 1987). Reports also exist that a male upon completing his parental duties may spawn with another female during any breeding season (Sigler and Sigler, 1987). For either or both of these reasons, it is perhaps best to speak of *I. punctatus* as likely having a behavioral tendency for serial or successive (rather than life-long) genetic monogamy.

Admittedly, our sample size of nests (5–6) is small (noodling is not easy!). To address whether the current finding of genetic monogamy in *I. punctatus* departs significantly from the more typical observation of genetic polygamy in most other nest-tending fishes, we considered previous genetic results on the 22 fish species summarized in Table 1 of Avise et al. (2002). By affording equal weight to each of these species (regardless of the original number of nests surveyed), the mean frequency of nests containing at least some half-sib progeny was 70%. Thus, if we were to have randomly sampled six nests from the pooled collection of such species, the probability that all six would consist exclusively of full-sibs is less than 0.001. From this line of reasoning, we can at least conclude that the apparent incidence of genetic monogamy in *I. punctatus* is unusually high among fishes surveyed to date.

We can also address the issue by considering the observed frequencies of nests containing at least some non-full-sib progeny within each of those same 22 species individually, and again calculating the probability that a random sample of six nests might have contained full-sibs exclusively. By this line of statistical reasoning, only four species would have yielded an outcome as extreme as that for *I. punctatus*. One involved the nest-tending *Micropterus salmoides*, which displays biparental care of progeny (DeWoody et al., 2000), and the other three involved syngnathid species with internal gestation by males: the pipefishes *Syngnathus scovelli* (Jones and Avise, 1997) and *Nerophris ophidion* (McCoy et al., 2001) and the seahorse *Hippocampus angustus* (Jones et al., 1998). Although the broods carried by the males of the two pipefish species contain full sibs only, females in those species are known to mate at least occasionally with several males (Jones et al., 2001; McCoy et al., 2001). Thus, among the 22 species genetically surveyed, only *M. salmoides* and *H. angustus* apparently have levels of genetic monogamy comparable to that of *I. punctatus*.

Among fishes with external fertilization, social monogamy has been reported primarily in species with large body size, seasonal breeding, and biparental care (Barlow, 1986). However, rarely has the monogamy suspected from field behaviors been critically addressed by genetic parentage analyses. Two notable exceptions involve a mouthbrooding cichlid (*Eretmodus cyanostictus*; Taylor et al., 2003) and a large-bodied species of nest-tending sunfish (the Largemouth Bass, *Micropterus salmoides*; DeWoody et al., 2000). In each of these species, both males and females contribute to offspring care, and in each case molecular markers have confirmed strong tendencies for genetic as well as social monogamy. The current study provides an even rarer report of genetic monogamy in a fish species with uniparental offspring care (Jones et al., 1998).

Many biological and environmental factors are likely to impact cuckoldry rates in nest-tending fishes (e.g., Mackiewicz et al., 2005). For exam-

ple, high nest densities and the presence of specialized sneaker or satellite males (Gross, 1996) undoubtedly tend to elevate cuckoldry rates, whereas low nest densities and high nest defensibilities probably serve to decrease rates of male cuckoldry (all else being equal). It is not known whether tactic-specialist sneaker males are present in *I. punctatus*. However, the typically low nest densities and the large body sizes of breeding males may well jointly contribute to nest defense and thereby lower the incidence of male cuckoldry in this species, which in turn would contribute to the tendency for genetic monogamy that our molecular data imply.

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(AT, FB, JCA) DEPARTMENT OF ECOLOGY AND EVOLUTIONARY BIOLOGY, UNIVERSITY OF CALIFORNIA, IRVINE, CALIFORNIA 92697-2525; AND (DLW) COLORADO COOPERATIVE FISH AND WILDLIFE RESEARCH UNIT, COLORADO STATE UNIVERSITY, FORT COLLINS, COLORADO 80523. E-mail: (AT) tatarenk@uci.edu. Send reprint requests to AT. Submitted: 21 Nov. 2005. Accepted: 10 May 2006. Section editor: J. M. Quattro.