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Molecular Evidence for Multiple Paternity in a Population of the Viviparous Tule Perch *Hysterocarpus traski*

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Abstract

Population density might be an important variable in determining the degree of multiple paternity. In a previous study, a high level of multiple paternity was detected in the shiner perch *Cymatogaster aggregata*, a species with high population density and a high mate encounter rate. The tule perch *Hysterocarpus traski* is phylogenetically closely related to *C. aggregata*, but it has relatively lower population density, which may result in distinct patterns of multiple paternity in these 2 species. To test the hypothesis that mate encounter rate may affect the rate of successful mating, we used polymorphic microsatellite markers to identify multiple paternity in the progeny arrays of 12 pregnant females from a natural population of tule perch. Multiple paternity was detected in 11 (92%) of the 12 broods. The number of sires per brood ranged from 1 to 4 (mean 2.5) but with no correlation between sire number and brood size. Although the brood size of tule perch is considerably larger than that of shiner perch (40.7 vs. 12.9, respectively), the average number of sires per brood in tule perch is much lower than that in shiner perch (2.5 vs. 4.6, respectively). These results are consistent with the hypothesis that mate encounter rate is an important factor affecting multiple mating.

Key words: *genetic parentage, mate encounter rate, microsatellites, pregnancy*

Female multiple mating (polyandry), which results in multiple sires contributing genetic material to particular broods (multiple paternity), is a taxonomically widespread phenomenon (reviewed in Avise et al. 2011). The evolution of female promiscuity and multiple paternity can be promoted by direct material benefits (e.g., fertilization assurance and nutrient acquisition) and indirect genetic benefits (e.g., higher offspring diversity and higher offspring viability), which often may be difficult to distinguish in natural populations (Jennions and Petrie 2000; Uller and Olsson 2008; Coleman and Jones 2011). In addition to benefits obtained through multiple mating, both theoretical and empirical studies suggest that mate encounter rate is also an important factor influencing levels of multiple paternity (Kokko and Rankin 2006; Uller and Olsson 2008).

The tule perch (*Hysterocarpus traski*) is a viviparous surfperch (Embiotocidae) native to the rivers and estuaries of California, and is the only freshwater surfperch (Baltz and Moyle 1982). Phylogenetically, it is closely related to the shiner perch (*Cymatogaster aggregata*; Bernardi and Bucciarelli 1999). Mating in the tule perch occurs from July to September, and females store sperm for up to 6 months before eggs are fertilized (Hopkirk 1962; Bundy 1977). Young are born during May–June when food is abundant (Moyle 2002). During the mating season, males apparently hold and defend territories, and females may mate with multiple males (Moyle 2002). Multiple paternity was documented in broods of tule perch (Phelps et al. 1995), but the limited variability of an allozyme marker in that earlier genetic study precluded firmer conclusions.

By analyzing highly variable microsatellite loci, high degrees of multiple paternity have been detected in 3 other surfperch species, all of which are marine: shiner perch (Liu and Avise 2011), black perch (*Embiotoca jacksoni*), and striped seaperch (*E. lateralis*; Reisser et al. 2009). These findings indicate that multiple paternity is generally common in

surfperch broods. In the embiotocid taxa previously studied, population densities were consistently high. For example, high abundances have been recorded for the shiner perch and black perch in suitable habitats, with 90.9 and 30.5 individuals per 10-min otter trawl, respectively (Yoklavich et al. 1991). Based on SCUBA dive survey data, the population density of black perch ranged from 3.1 to 5.6 adult individuals per 100 m^2 on a southern California rocky reef and 0.8 to 3.2 individuals per 100 m^2 within 2 m of the bottom in Monterey Bay (Miller and Geibel 1973; Pondella et al. 2002). In Monterey Bay, the population density of striped surfperch was even higher than for black perch, with 2.1–7.4 individuals per 100 m^2 within 2 m of the bottom (Miller and Geibel 1973). By contrast, using similar sampling gear and procedures across the years 1980–2010, the population abundance of tule perch in the Suisun Marsh ranged from only 0.2 to 6.2 individuals per 5 min tow with an otter trawl (Schroeter and Moyle 2004; O'Rear and Moyle 2011). Although the methods for estimating densities are not strictly comparable, they suggest that population densities of the other surfperch species are considerably higher than for tule perch in the Suisun Marsh (Schroeter and Moyle 2004; O'Rear and Moyle 2011). In addition, all 3 species previously studied breed in large aggregations (Wiebe 1968; Froeschke et al. 2007), and this factor should further increase the effective rate of mate encounters. Furthermore, water transparency is low in Suisun Marsh, with average secchi depths being generally less than 30 cm (Schroeter and Moyle 2004) and therefore much lower than in the coastal California waters inhabited by the other surfperch species (Tibby and Terry 1958). Visual signals are known to be important during courtship communication of surfperches (De Martini 1969; Cummings 2007). If population density and water transparency are important variables influencing effective rates of mate encounter and multiple paternity, we would expect a much lower degree of multiple paternity in tule perch than in the other surfperches previously analyzed.

Here, we developed highly variable microsatellites to investigate genetic paternity in broods of pregnant females obtained by trawl field investigations. This study allows us to address 2 main questions: 1) What are the frequency and degree of multiple paternity in the tule perch? 2) How do patterns of multiple paternity in this species compare with those of other surfperches previously assayed?

Materials and Methods

Sample Collection

In May 2010 and April 2011, 5 (TP01–TP05) and 7 (TP06–TP12) gravid females of tule perch were collected, respectively, in Suisun Marsh, CA, during a sampling program conducted by University of California at Davis. To isolate microsatellites, fin clips of 16 adults (sex unknown) were also collected in Suisun Marsh in January 2010. Standard length (mm) was measured for each female, ovaries were then dissected, and embryos counted. Fin clips of adults and whole embryos were preserved in a saline solution (20% dimethyl sulfoxide, 0.25 M EDTA, saturated with NaCl, pH 8.0) (Seutin et al. 1991) for DNA analysis.

Microsatellite Development

Microsatellites were isolated from a single specimen of tule perch after an enrichment protocol described by Hamilton et al. (1999) and modified by Hauswaldt and Glenn (2003). In brief, 4 μg of genomic DNA were digested with a restriction enzyme that forms blunt ends (BstUI; New England Biolabs). Digested fragments were ligated to double-stranded SuperSNX24 linkers (forward 5'-GTTTAGGCCTAGCTAGCAGAATC-3'; reverse 5'-pGATTCTGCTAGCTAGGCCTTAAACAAAA-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 procedure of hybridization, bead capture, wash, and recovery was repeated twice to ensure better enrichment for microsatellite repeats. The recovered DNA fragments were next amplified via PCR using linkers as priming sites, and cloned using the TOPO TA Cloning Kit (Invitrogen Life technologies, Carlsbad, CA). 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 (version 3.1, Applied Biosystems, Foster City, CA). Sequencing was conducted on an ABI 3130xl Genetic Analyzer equipped with 50 cm capillaries. Primers flanking the microsatellite repeat regions were designed using Primer Premier version 5.00 (PREMIER Biosoft International). Primers were optimized and checked for polymorphisms using a sample of 28 adults. Observed and expected heterozygosities were calculated, and deviations from Hardy–Weinberg equilibrium were examined using an exact test based on a Markov chain method with 5000 dememorisation steps and 100 000 Markov chain steps (Guo and Thompson 1992) for each locus. The presence of genotypic disequilibrium between all pairs of loci was tested using the likelihood ratio test. All analyses were conducted for the 28 adults by using Arlequin version 2.0 (Schneider et al. 2000). Expected exclusion probabilities (Dodds et al. 1996) for each locus and across all loci were calculated with GERUD 2.0 (Jones 2005). The presence of null alleles for each locus was checked using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). A total of 6 highly polymorphic loci (5 with dinucleotide repeats and 1 tetranucleotide) were chosen for the current study.

Microsatellite Genotyping

Genomic DNA was extracted from fin clips of adults and posterior parts of embryos using a proteinase K digestion and phenol/chloroform/isoamyl extraction procedure (Milligan 1998). Tailed PCR was used to produce fluorescently labeled DNA fragments (Boutin-Ganache et al. 2001).

A short oligo M13Rev (5'-GGAAACAGCTATGACCAT-3') was added to the 5'-end of 1 primer in each pair. A fluorescently labeled M13Rev (FAM, HEX, or NED) was included in the PCR, resulting in a labeled product for detection. All loci were amplified separately on a Mastercycler (Eppendorf) in a 10 μL reaction containing about 50 ng genomic DNA, 0.2 mM of each dNTP, 1× GoTaq buffer (Promega, Madison, WI), 0.2 μM labeled M13Rev primer and locus-specific primer without tail, 0.02 μM locus-specific primer with M13Rev tail, and 0.25 U *Taq* DNA polymerase (Promega). Thermal cycling parameters for all amplifications were 95 °C for 3 min, then 35 cycles each at 95 °C for 20 s, 52 °C for 20 s, and 72 °C for 30s, followed by 1 cycle of final elongation at 72 °C for 10 min. Amplified products were diluted 15- to 20-fold and pooled, and 1 μL of the pool was mixed with 10 μL of deionized formamide and 0.3 μL of GS500 size standard (Applied Biosystems). Samples were denatured for 4 min at 95 °C and electrophoresed on an ABI PRISM 3100 Genetic Analyzer. Allele scoring was performed using GENEMAPPER software version 4.0 (Applied Biosystems). Scoring was repeated for 16 random individuals at all 6 loci to estimate scoring error (but identical genotypes were obtained for all replicates).

Paternity and Statistical Analysis

Paternity inference was conducted using COLONY 2.0, which implements a maximum likelihood method that takes into account maternal information and estimated allele frequencies in the population (Wang 2004; Wang and Santure 2009; Jones and Wang 2010). COLONY evaluates genetic parentage by partitioning offspring into full-sib groups according to likelihood scores assuming Mendelian segregation and no maximum limit on the numbers of contributing parents. The configuration with maximum likelihood is searched by a simulated annealing algorithm. Thus, the number of sires contributing to each female's brood and the reproductive skew among males could be determined. A linear regression test for a correlation between brood size and female standard length was performed. To assess whether larger broods were more likely to contain offspring from each of the males with whom a female had mated, a linear regression test for correlation between brood size and the number of sires was performed.

Results

Female Size and Fecundity

A total of 12 pregnant females with standard lengths ranging from 96 to 155 mm were collected. Average brood size was 40.7 (±6.63) individuals (ranging from 15 to 91 individuals across the 12 broods). Stages of gestation varied among broods, but within a brood there was little range in the stage of embryonic development. Brood size was positively correlated with female standard length (r^2 = 0.80, degrees of freedom [df] = 11, $P < 0.001$).

Microsatellite Markers

All 6 microsatellite loci were highly variable, displaying from 13 to 19 alleles per locus (Table 1). Observed and expected heterozygosities were high for all loci and ranged from 0.86 to 0.96 (Table 1). No evidence of genotypic disequilibrium between loci was detected and all loci were in Hardy–Weinberg equilibrium. These loci were highly informative for parentage analysis, with expected exclusion probabilities (under the one-parent-known model) ranging from 0.75 to 0.81 for each locus and >0.999 for all 6 loci combined (Table 1). Microchecker did not find any evidence for the presence of null alleles at any locus.

Genetic Paternity

Genotypes at 6 microsatellite loci were determined for 28 adults and a total of 428 embryos. No null alleles or maternally derived *de novo* mutations were detected. For each brood, the best (maximum likelihood) inferred configuration consisting of full-sib families is shown in Table 2. Multiple paternity was

Table 1 Characterization of 6 polymorphic microsatellite loci for 28 individuals of tule perch

Locus	Primer sequence (5′-3′)	Repeat motif	Number of alleles	Size range (bp)	H_o	H_e	Exclusion probability
Htra02	[a]F: *Ned*-GGGTCAAAATCACTCTGCTA R: GTTTGTAAGCGTTTCACTG	$(CA)_{18}$	13	128–164	0.857	0.892	0.751
Htra22	[a]F: *Hex*-GGGGCATTCCCTAGTTTTGG R: AGGGGTGACCCAGTCCAGAT	$(GT)_{20}$	18	128–188	0.929	0.923	0.813
Htra25	[a]F: *Fam*-CACCAGACCTCTTCACAAAC R: CTGGAGAAGGCATAAAGAAA	$(GT)_{21}$	19	192–262	0.893	0.908	0.790
Htra26	[a]F: *Fam*-GGAAACTGGTCCTCACAAAG R: CTTCATCGCAGCGTGGCAAT	$(CA)_{29}$	17	144–182	0.857	0.926	0.817
Htra49	[a]F: *Hex*-GTTTGTTGACATGACAGATGCTA R: GTTCAGTGCCAGCGGTGTTG	$(TATC)_{31}$	18	267–347	0.929	0.926	0.818
Htra56	[a]F: *Ned*-GGTTGTGATGGACCA R: CAGCATGGCGTGACAATTTC	$(GT)_{24}$	17	198–270	0.964	0.924	0.814
Over all loci	N/A	N/A	N/A	N/A	0.904	0.917	0.999

[a]The complete sequence of the primer includes M13 Reverse tail (5'-GGAAACAGCTATGACCATG-3') at its 5'-end. M13 Reverse oligo labeled with Fam, Hex, or Ned was used in the PCR reaction.

Table 2 Multiple paternity for 12 broods of tule perch from Suisun Marsh

Family	Brood size (*n*)	Standard length (mm)	Number of embryos analyzed	Number of sires	Sire1	Sire2	Sire3	Sire4
TP01	15	114	15	2	11	4		
TP02	23	108	23	2	22	1		
TP03	34	107	34	4	19	7	2	6
TP04	53	128	53	2	19	34		
TP05	22	115	22	1	22			
TP06	91	155	60	4	24	16	12	8
TP07	74	150	56	2	47	9		
TP08	54	140	43	3	40	2	1	
TP09	27	100	27	2	25	2		
TP10	28	96	28	4	17	4	4	3
TP11	31	105	31	2	19	12		
TP12	36	110	36	2	23	13		

Shown are brood size, standard length of the female parent, number of sires per brood, and number of offspring sired by up to 4 putative males as inferred by COLONY version 2.0.1.1 (Wang 2004).

detected in 11 (92%) of the 12 broods. The number of sires per brood ranged from 1 to 4, with an average of 2.5 sires per brood (Table 2). The number of sires per brood was not significantly correlated with brood size ($r^2 = 0.10$, df = 10, $P = 0.31$).

Discussion

Our microsatellite analyses revealed a frequency of multiple paternity in tule perch (92%) that is much higher than the previous estimate (21%) for this species based on an allozyme marker (Phelps et al. 1995). Microsatellite analyses have also indicated high frequencies of multiple paternity in broods of 3 other embiotocid species: shiner perch (96%) (Liu and Avise 2011), black surfperch (100%), and striped seaperch (100%) (Reisser et al. 2009). These genetic analyses indicate that polyandry is an extremely common phenomenon in surfperches, placing these fish among internally brooding species with the highest incidences of multiple mating (Coleman and Jones 2011).

Although the brood size of tule perch (40.7) is much higher than that (12.9) of the closely related shiner perch ($P < 0.01$; one-tailed *t*-test), the average number of sires per brood in tule perch (2.5) is much lower than in shiner perch (4.6) ($P < 0.01$; one-tailed *t*-test). In addition, the average number of sires per brood in tule perch is lower than those in black surfperch ($P = 0.02$; one-tailed *t*-test) and in striped seaperch (although not significant, $P = 0.08$; one-tailed *t*-test). These findings are inconsistent with the hypothesis that clutch size constrains successful mate numbers in species with extended parental care (Avise and Liu 2010; Avise et al. 2011), but they are generally consistent with a "logistical constraint" hypothesis stating that mate encounter rates are a proximate factor that can affect the degree of polygamy (Avise and Liu 2011).

The high frequency of multiple paternity in tule perch and other surfperches may be explained by some near-universal fitness benefits that can come from multiple mating (Liu and Avise 2011). Compared with the other surfperch species studied, the population abundance of tule perch is low (Schroeter and Moyle 2004; O'Rear and Moyle 2011). Furthermore, surfperches display a variety of colors (e.g., orange, blue, silver) during the breeding season and visual signal plays an important role during courtship communication (De Martini 1969; Cummings 2007). Consequently, the very high turbidities in Suisun Marsh could limit the range of conspecific detection during the tule perch breeding season. Thus, the low population density and water transparency in Suisun Marsh could result in effectively low mate encounter rates with a concomitant reduction of the number of sires per brood in this species. However, a relatively high population density (Miller and Geibel 1973; Yoklavich et al. 1991; Pondella et al. 2002) and high degree of water transparency (Tibby and Terry 1958) could in part be responsible for the higher number of genetically detected sires per brood in the other embiotocid taxa studied.

Studies of some other taxa also have shown a positive relationship between levels of multiple mating and population density. For example, a positive correlation between rates of multiple paternity and rates of mate encounter were reported among natural populations of the poeciliid fish *Heterandria formosa* (Soucy and Travis 2003). Mobley and Jones (2007) found that the rate of male multiple mating in the dusky pipefish (*Syngnathus floridae*) was higher in populations with greater densities. Populations of the land snail *Arianta arbustorum* with the highest density of adults showed the highest levels of multiple paternity, whereas snails in the population with the lowest density exhibited the lowest value of multiple paternity (Kupfernagel et al. 2010). In the marine snail *Littorina saxatilis* with high population density (100–1000/m^2), extreme levels of multiple paternity were detected, with 15–23 males having sired the offspring of each female (Panova et al. 2010). The pattern of multiple paternity across reptilian taxa also indicates that the degree of multiple paternity is generally higher in species with higher mate encounter rates (Uller and Olsson 2008). Thus, all of these earlier findings as well as those in the current study are consistent with the hypothesis that rate of mate encounter is an important factor affecting the degree of genetically successful multiple mating.

Factors affecting male mate choice can also limit level of female multiple mating. Sperm competition theory predicts

that males should be sensitive to sociosexual cues that provide information about the sperm competition risk (SCR) and correspondingly adjust their mating tactics in a manner that maximizes their reproductive success (Wedell et al. 2002). Males of some poeciliid fishes were reported to avoid SCR by determining a female's mating status using chemical cues or by directly observing sexual interactions between rival males and potential mates (Dosen and Montgomerie 2004; Wong and McCarthy 2009; Ziege et al. 2009; Jeswiet et al. 2011). Also, mating behaviors can be affected by the "audience effect" where male's expression of mating preferences is modified depending on the presence or absence of another bystanding male to prevent the phenomenon called "male mate choice copying" (Plath and Schlupp 2008). The modulation of sperm investment by males can sometimes result in sperm limitation for females and limit multiple paternity (Wedell et al. 2002; Plath and Bierbach 2011). However, these hypotheses require good visual conditions, so both hypotheses are less likely for tule perch in the Suisun Marsh because of turbid waters and low population density.

In the present study, only one population of tule perch from Suisun Marsh was analyzed. Tule perch is distributed in 3 drainages in central California and there are striking differences in life-history characteristics and environmental conditions among different populations (Baltz and Moyle 1982). To draw general conclusions about the entire species and address how ecological and demographical variables influence mating behavior, it would be very interesting to compare the levels of multiple paternity among different geographic populations of tule perch when possible.

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