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Parentage and Nest Guarding in the Tessellated Darter (*Etheostoma olmstedi*) Assayed by Microsatellite Markers (Perciformes: Percidae)

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Parental investment as manifested through extended parental care of young presumably enhances the reproductive success of the custodial parent. In the Tessellated Darter (*Etheostoma olmstedi*), the primary caregivers are breeding males on the nest. However, prior field observations on nesting darters seem suggestive of behaviors that are more difficult to interpret evolutionarily. These include tending clutches that may have been fertilized by other males and appropriating nests from smaller courting males. To address such possibilities genetically, we assayed six microsatellite loci in 16 nest-tending males and the embryos from their associated clutches. In most cases, a guardian male had sired nearly all of the embryos in his nest. However, in one nest, a guardian male had been cuckolded, and in two other nests, an attendant male guarded embryos that were not his own presumably resulting from nest takeovers. From direct genotypic counts, a mean of at least 3.2 mothers contributed to the progeny in a nest, and computer simulations suggest that the true maternal number may be substantially higher.

THE spawning behaviors of fishes are diverse and often include intense reproductive competition via such strategies as group spawning, male-male conflict, and sperm competition (Taborsky, 1994). However, only a few molecular studies have documented the genetic outcomes of these varied reproductive tactics in multiple nests or clutches (e.g., DeWoody et al., 1998; Jones et al., 1998; Kellogg et al., 1998). Herein, we provide a genetic perspective on the reproductive behavior of the Tessellated Darter, *Etheostoma olmstedi*.

Breeding behavior for this species is complex, with nest site availability apparently a critical factor in reproductive success (Atz, 1940; Constantz, 1979, 1985). ''Bourgeois'' nest-attendant males (Taborsky, 1997) defend flat-bottomed stones that serve as spawning sites, and females preferentially oviposit eggs directly on the underside of bare stones with greater surface area (Constantz, 1985). Fathers often abandon established nests containing their offspring, presumably to search for better nesting situations (Constantz, 1985). Nest-attendant males also may ''roam'' in apparent attempts to seize fertilizations from more submissive males. If the small displaced male had been courting a female, she often resumes courtship with the larger male, such that in effect large cruising males usurp fertilizations from smaller courting males (Constantz, 1979). Displaced males then may assume the behavior and appearance of a receptive female and attempt to reenter the nest, presumably attempting to sneak fertilizations from the dominant male (Constantz, 1979).

Fertilization success of these various reproductive behaviors is uncertain in the absence of genetic data. We have used a suite of six microsatellite loci to study parentage, nest guarding, and cuckoldry in a population of tessellated darters from the southeastern United States. In addition, we address mutation rates and use computer simulations of our data to estimate the number of parents contributing to a nest.

MATERIALS AND METHODS

Field collections and laboratory methods.—In March 1998, when water temperatures ranged from 14–17 C, we collected 16 distinct egg masses (''nests'') and their associated adult male guardians from a 2.5-km section of Fourmile Creek, a tributary of the Savannah River near Barnwell, South Carolina. Adults were electrofished, and egg masses were collected from the undersides of stones and woody debris. Each adult was assigned a unique voucher number and preserved in a solution of 0.25M EDTA, 20% DMSO, and saturated NaCl. In the laboratory, embryos from each egg mass were classified as early embryos, middle embryos, near hatchlings, or hatchlings. Embryos of different developmental stages usually were present in a nest, and when used in conjunction with the genetic data, these stages often facilitated interpretations of reproductive behavior. Embryos were sampled randomly from within each developmental stage found in a nest, and sample sizes were equitable among the various developmental stages.

DNA from the darter samples was isolated us-

ing a modified fly buffer protocol described in Gloor and Engels (1992). Briefly, embryos were rinsed in distilled water and then dissected from the surrounding egg yolk. Dissected embryos then were incubated in 50 μl of buffer (10 mM Tris 8.0, 1 mM EDTA pH 8.0, 25 mM NaCl, 0.3 mg/ml proteinase K) for 30 min at 55 C, followed by a 2-min incubation at 95 C. After a brief centrifugation, 3 μl of the supernatant was used as template for PCR amplifications.

Darter microsatellites were cloned from a genomic library using standard protocols (Choudhary et al., 1993). The library was screened with various di-, tri-, tetra-, and penta-nucleotide repeat motifs. Positive clones were isolated and sequenced, and PCR primers were designed to amplify the region containing the microsatellite. All PCR amplifications (except for locus *EO4*) employed Promega's *Taq* DNA polymerase (0.5 unit/reaction) and buffer. Locus *EO4* was amplified using 1 unit of *Taq* per reaction and a 10× buffer of 0.5 M KCl, 0.1 M Tris 8.0, 25 mM $MgCl_2$, and 0.5 mg/ml BSA. Two pairs of loci (*D1/EO9* and *EO6/EO12*) were multiplexed; the other two loci (*EO4/EO7*) were amplified individually.

Microsatellite loci initially were screened for variability using radioactive methods (e.g., Choudhary et al., 1993). Primers for loci that were consistently scorable (and polymorphic) were dye-labeled for automated detection. Fluorescent PCR products (0.3–0.5 μl) were electrophoresed on an ABI 377 sequencer, and genotypes were determined using the manufacturer's software. The fluorescently tagged markers were resolved on two sets of gels, one with loci *EO6/EO7/EO12* and the other with loci *D1/EO4/EO9*.

Statistical issues and analyses.—Exclusion probabilities were calculated according to Selvin (1980), Chakraborty et al., (1988), and Dodds et al., (1996). Genotypes were scored in each embryo and nest-attendant male. From this information, maternal gametes and haplotypes (i.e., ''gametotypes''; DeWoody et al., 2000) were deduced by subtraction for each embryo. The number of distinct gametotypes found in the entire progeny array (which usually proved to be composed of full-sibs and half-sibs) then was tabulated. Computer simulation programs were used to determine the number of embryos per nest required to detect all maternal genetic contributions and to estimate how many mothers contributed to a half-sib progeny array. These simulations are described in detail by DeWoody et al. (2000) and are merely summarized here.

All simulations utilized the empirically determined allele frequencies at each locus and Hardy-Weinberg equilibrium to generate a pseudopopulation of adult genotypes. These genotypes then were sampled randomly and used as progenitors. Each progeny array was generated by ''mating'' the progenitors using Mendelian inheritance coupled with a uniform distribution of reproductive success among females. ''Embryos'' then were sampled randomly from each full- or half-sib array until each progenitor gametotype was recovered. Distributions of the number of gametotypes were generated for simulated nests (batches of embryos) having between two and 15 mothers. Results were sorted, and the most likely number of parents to contribute to a nest was recorded. For example, one trial with four different mothers may result in 24 different gametotypes represented among the progeny array, whereas the next trial may find 26 gametotypes resulting from four different mothers.

Results

Genetic markers.—Eleven positive clones were isolated and sequenced from the darter genomic library. All of these contained microsatellites, nine involving $(AC)_n$ repeats and two involving $(AG)_n$. Six of the di-nucleotide loci (Appendix) were polymorphic and consistently scorable (Fig. 1). Each locus was amplified and scored in more than 600 individuals (Fig. 2).

In a sample of 36 presumably unrelated adults from the population, the numbers of alleles per locus ranged from three to eight, with allele frequencies shown in Figure 1. As gauged by a lack of significant departures from Hardy-Weinberg equilibrium in the adult population sample [Appendix; exact test of Guo and Thompson (1992) in GENEPOP (Raymond and Rousset, 1995)], null alleles were not present in appreciable frequency at these six loci. However, a null allele might explain an anomalous gel pattern for nest 5. There, at the *EO12* locus only, some of the embryos did not inherit an ''allele'' corresponding to the single gel band of the apparently homozygous guardian male (who was their biological father as gauged by the other five loci). Otherwise, null alleles did not compromise the parentage analyses that are the primary focus of this study.

Assuming (as usually proved true) that an attendant male fathered the embryos in his nest, the combined exclusion probability (Chakraborty et al., 1988) for all six loci was 0.90. In practice, this probability is an underestimate of the exclusion power, because not every allele

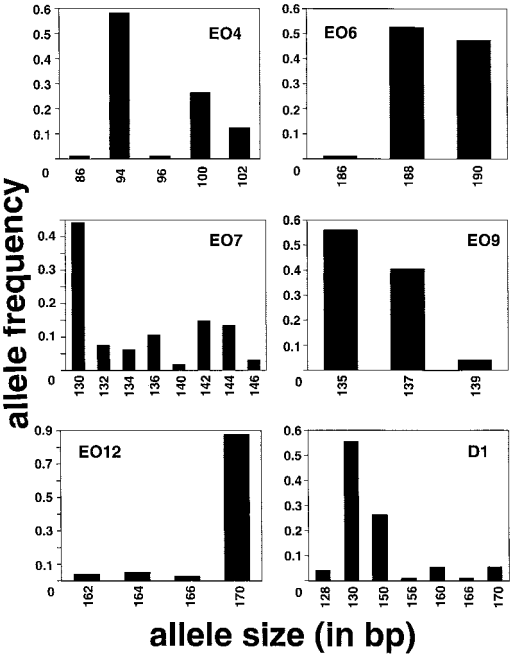

Fig. 1. Allele frequencies at each of six microsatellite loci in 36 presumably unrelated adult darters taken from the study site.

present in the darter population is represented in our sample of adult genotypes. In many cases, maternally derived alleles were detected in progeny arrays, but those same alleles were not detected in the adult population. Thus, a more exhaustive population survey would reveal even more allelic diversity and increase the exclusion probabilities. In any event, given this level of variation and assuming that about 10 females on average contributed equitably to a typical nest, computer simulations (DeWoody et al., 2000) indicate that approximately 40 embryos per nest should be genotyped to detect at least one gamete from each mother. If fewer than 10 mothers normally contributed to a nest, smaller samples of offspring should suffice. We genotyped a mean of 38 embryos per nest.

Paternity.—The sampled progeny arrays within 13 of the 16 nests studied (81%) were sired exclusively by the attendant male (Table 1). This indicates a great preponderance of reproduction by the guardian or bourgeois males. However, in each of two other nests (4 and 6), inspection of the multilocus genotypes revealed that more than 50% of the offspring were sired by males other than the guardian. In each case, this genetic deduction was corroborated by independent evidence on brood developmental phase.

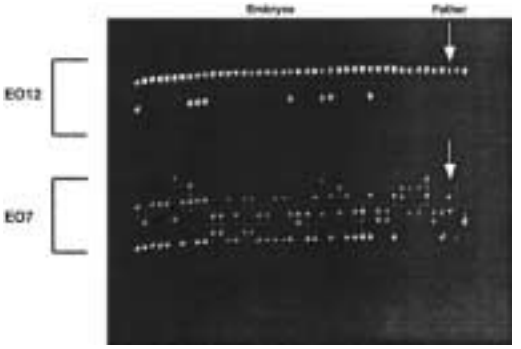

Fig. 2. Gel image illustrating genotypes at two of the six loci used in the current study. The two loci were labeled with distinct dyes such that locus *EO12* fluoresced blue and locus *EO7* appeared green.

For example, embyros in nest 4 were of two distinct developmental stages. The guardian male sired all 22 of the younger embryos surveyed, whereas another (unsampled) male sired all 33 scored members of an older developmental cohort. Furthermore, from genetic evidence on maternity (as described later), each male who contributed to this nest had fertilized eggs from at least two females, one of whom was not shared between the two fathers. Thus, at least three females spawned in nest 4, and the nest guardian at the time of sampling likely had usurped the nest from an earlier male who sired the first clutch.

Nest 6 also contained embryos of two distinct developmental stages. Again, the attendant male sired all 10 of the less-developed cohort of embryos, suggesting a nest takeover. However, unlike the case for nest 4, genetic analyses of the older cohort suggest that at least two males sired those embryos. Apparently, either (1) the ''initial'' nest-guarding male was cuckolded by another male; or (2) he usurped the nest from an even earlier male, only to have the nest taken over by the third male (our collected attendant).

The remaining nest (8) was unusual in that it was situated close (< 0.5 m) to another nest (7). Two adult males were captured in the near vicinity. One of these males displayed a genotype inconsistent with fatherhood for any of the embryos in either nest. The other male proved to be the father of all embryos in nest 7, and he also could have sired 10 of the embryos from nest 8. If that were true, however, two additional (uncaptured) males would be required to account for the remaining genotypes in nest 8. Alternatively, genotypes can be constructed for two hypothetical males (other than those captured) that could account for all of the geno-

TABLE 1. SUMMARY OF DARTER BREEDING BEHAVIOR AS ASSESSED BY MOLECULAR MARKERS. In three nests where the attendant male did not sire all sampled offspring (4, 6, and 8), no estimates of maternal numbers are provided because the maternal gametic contributions could not be differentiated from paternal ones. 95% confidence intervals are shown in parenthesis.

Nest ID	embryos in nest	Paternity by attendant male	Embryonic stages	Min no. of mothers	Est. no. of moms[a]
1	2976	53 of 53	2	4	8.9 (3, 15)
2	580	20 of 20	1	2	2.1 (1, 4)
3	1460	40 of 40	2	3	11.9 (6, 15)
4	1805	22 of 55[b]	2	—	—
5	3964	42 of 42	2	4	12.6 (7, 15)
6	1360	19 of 40[b]	2	—	—
7	1714	30 of 30	3	4	6.5 (1, 13)
8	2223	10 of 43[b]	3	—	—
9	2616	27 of 27	3	3	5.0 (1, 11)
10	28	14 of 14	1	1	full-sibs[c]
11	2040	40 of 40	3	4	11.0 (4, 15)
12	596	46 of 46	2	3	12.0 (5, 15)
13	1787	37 of 37	3–4	4	11.4 (5, 15)
14	760	46 of 46	1	4	12.5 (7, 15)
15	1172	31 of 31[d]	1	3	9.7 (3, 15)
16	671	46 of 46	1	3	7.3 (2, 14)

[a] Mean of the multilocus simulations in DeWoody et al. (2000).
[b] See text.
[c] Entire working collection surveyed; nest consisted of only full-sibs and thus there was only one mother.
[d] Two embryos were inconsistent with the guardian male's genotype at a single locus; thus either a mutation or a cuckoldry event occurred.

types in nest 8. In that case, one of these hypothetical males sired 35 of the 43 embryos assayed, and the second male sired the other eight offspring. In any event, at least two males were sires for nest 8. Furthermore, each male produced embryos of mixed developmental stages; thus, one likely explanation is that cuckoldry was involved (perhaps in conjunction with a nest takeover).

Maternity.—As evidenced by direct counts of deduced maternal gametotypes, in only one case (the unusually small nest 10) did all embryos share the same mother. Within each of the remaining 15 nests (94%), the sample of embryos had more than one female parent. From direct genotypic counts, on average at least 3.2 mothers spawned successfully in each nest, and computer simulations suggest that the true number often is more than eight (Table 1). Winn (1958a,b) found that, in the presumptive sister-species *E. nigrum* (and in other closely related taxa), clutch sizes range from 30 to 200 eggs and the mean is around 100. Given that our mean nest size was approximately 1600 embryos, the computer simulations probably more accurately reflect the true number of mothers per nest than do estimates based strictly upon the minimum number of maternal gametotypes.

Ideally, we would like to know the exact numbers of mothers and their precise relative contributions to the pool of embryos within each nest. Although maternal gametic contributions (and thus the minimum number of mothers) were obvious from the genotypes of half-sib embryos, precise reconstructions of maternal genotypes usually proved impossible for two reasons. First, alleles at marker loci are often shared by putative mothers (and by the known father), and second, many more embryos must be sampled per nest to permit firm reconstructions of multilocus maternal genotypes when several females are involved. Theoretically, the minimum such sample size can be estimated (n^* in DeWoody et al., 2000), but in practice such intensive sampling is intractable when more than a few nests are involved. Thus, we were unable to determine whether the same mother spawned in more than one nest.

DISCUSSION

In fishes as in other organisms such as birds, a distinction must be made between the social mating system and the genetic mating system. Only the latter reflects the realized pattern of inheritance. In tessellated darters, field biologists have reported a variety of spawning and nesting behaviors (Constantz, 1979, 1985). Our genetic data corroborate that notion and permit quantitative appraisals of the relative con-

TABLE 2. INCLUSION PROBABILITIES FOR TWO EMBRYOS (A AND B) FROM NEST 4. Both embryos are consistent with the genotype of the putative father (shown in the first row) at five of the six loci (*EO4, 6, 7, 9,* and *12*). However, one locus (*D1*) is inconsistent with paternity by the attendant male. The specific inclusion probabilities (Selvin, 1980) are represented by P_i, whereas P'_i represents the discounted inclusion probability (see text).

Locus	*EO4*	*EO6*	*EO7*	*EO9*	*EO12*	*D1*	$\prod_{i=1}^{6} P_i$
Dad?	94/102	189/189	130/134	235/235	170/170	152/152	
A	94/94	189/189	134/142	235/237	170/170	132/132	
P_i	0.826	0.776	0.370	0.998	0.985	0.806	0.188
P'_i	0.583	0.522	0.206	0.959	0.878	0.558	0.029
B	94/102	189/191	130/130	235/237	164/170	132/132	
P_i	0.915	0.999	0.688	0.998	0.996	0.806	0.504
P'_i	0.709	0.990	0.441	0.959	0.932	0.558	0.154

tributions of various mating behaviors to reproductive success in this study population.

Mutations or exclusions?—When a nest guardian is excluded as the father of some or all of the progeny in his nest at several highly polymorphic loci, the inference of exclusion is secure. However, when a single locus or a low level of polymorphism is involved, a potential exclusion must be weighed against the alternative possibility that progeny received a new mutation from the guardian male who was their true father. The probability that a new mutation accounts for the aberrant genotype is simply the mutation rate, estimates of which for microsatellite loci range from about 0.01 to 0.00001 (Ellegren 1995; Jin et al., 1996; Primmer et al., 1996, 1998), with a mean of perhaps 0.001. These values are to be contrasted with the probability that another male in the population sired the ''aberrant'' offspring (i.e., that the guardian was cuckolded, or a nest takeover occurred). This probability is a function of the allele frequencies in the population and of the specific genotypic configurations of the offspring.

To address this latter probability, two related questions can be posed. First, what is the frequency in the population of all males whose genotypes are compatible (in this case, at all six loci) with being the father of the particular offspring in question? For a population in Hardy-Weinberg equilibrium, this probability (p_i) is given by the product across loci of the paternal inclusion probabilities for specific offspring genotypes. Second, what is the probability that these genotypically compatible males contributed the aberrant allele to that embryo? This latter probability (p'_i) will be lower than the former because it discounts by 50% the inclusion probability for each candidate male who is heterozygous for the allele in question.

Table 2 illustrates such calculations. Shown are the genotypes for two embryos and from the male attending nest 4. At five of the six loci, the genotypes in embryos are consistent with the guardian male being the father. In the right-hand column are probabilities that a random cuckolder or previous nestholder truly sired these embryos, as calculated by the two procedures mentioned above. In this case, the probabilities of cuckoldry (or nest takeover) are considerably higher than the mutation rate. For this nest, the likelihood that a nonattendant male sired the genotypically aberrant offspring is bolstered by the fact that all (except one) of the excluded embryos were at a different (older) developmental stage than those presumably sired by the nest guardian.

In light of the above discussion, only two likely mutation events were observed in our data. Two embryos from nest 15, both displaying an identical genotype at locus *EO4*, were inconsistent with the guardian male's genotype at that locus only. Specific inclusion probabilities (p'_i-values) were 0.005 and 0.008 respectively, suggesting that cuckoldry was about as likely as mutation. Thus, two possibilities exist: (1) a few offspring in the nest arose from a cuckoldry event; or (2) a mutation arising in the germ line of the attendant male was transmitted to a small cluster of embryos (Woodruff and Thompson, 1992; Jones et al., 1999). If, indeed, only two mutations were detected among the 610 embryos scored, mutation rates at locus *EO4* are about 3.2×10^{-3}. By the same reasoning, mutation rates at the other five loci are less than 1.6×10^{-3}.

One key point from such single-locus considerations is that average exclusion values should not be used to distinguish between a cuckoldry event and a new mutation as the source of an aberrant allele in a progeny array. Rather, these competing possibilities should be compared

case by case using the specific exclusion probability for the offspring in question.

Singleton sires and multiple dams.—By genetic evidence, 523 of the 610 embryos (86%) assayed were sired by a bourgeois male who was guarding the nest at time of capture. Indeed, in 13 of the 16 nests surveyed, the guardian male apparently sired all of the embryos sampled. However, modest numbers of embryos were the product of two other routes to paternity. First, 54 embryos in the study (9%) were tended by ''nest-takeover'' males who presumably had usurped nests from the true biological fathers of these offspring. There were two such takeovers, and in each case, more than 50% of the embryos sampled had been sired by a male other than the current attendant. Second, the other 33 embryos (5%) in the study were the probable result of cuckoldry at one of the nests (8) such that they were sired by a male who sneaked fertilizations.

The occurrence of nest takeovers may reflect a limited availability of nest sites to males, and/or purposeful actions by males to attract females. Observational evidence is consistent with both scenarios. Nest site availability is known to be a limiting factor in darters (Constantz, 1985); and females of many species prefer males whose nests already contain eggs (e.g., Ridley and Rechten, 1981). Indeed, on the tips of their spines and rays, males in several darter species (including *E. olmstedi*) display fleshy masses that mimic eggs, and it has been proposed that these features evolved because males benefit in terms of mating success from having (or appearing to have) eggs in their nest (Page and Bart, 1989). Another possibility is that some nests are simply abandoned by the guardian male and appropriated by subsequent males who then spawn with new females.

With respect to maternity, the genetic data reveal that a guardian male typically spawned with multiple females. From direct genotypic counts of deduced maternal gametic types in the half-sib broods, the collection of embryos within a nest had a mean of 3.2 mothers. However, because of finite genetic sampling, these numbers are minimum estimates (Nason et al., 1996; DeWoody et al., 1998; Kellogg et al., 1998).

Various methods have been developed to estimate the actual number of parents contributing to a half-sib progeny array (Harshman and Clark, 1998; Marshall et al., 1998). Here we employed a computer-simulations approach designed expressly for situations in which large numbers of full-sib and half-sib embryos have been sampled from a nest at each of several highly polymorphic loci (DeWoody et al., 2000). The programs sample repeatedly from a simulated population that has identical genetic parameters (number of loci, alleles per locus, and allele frequencies) to the study population. The output consists of estimates of the most likely number of parents who contributed to the progeny in each nest. As applied to the current situation, these simulations suggest that as many as 12 different mothers may contribute to a single nest (Table 1).

Ichthyologists often have used the number of developmental phases of embryos in a nest to estimate how many mothers contributed to the progeny array (see Dominey and Blumer, 1984). However, our data indicate that the developmental stage of embryos is not an infallible indicator of maternal counts. For example, all embryos in nest 14 were of the same developmental stage, yet the genetic data show that at least four mothers contributed to that nest (Table 1). Similar reports exist for the Redbreast Sunfish (DeWoody et al., 1998). There, individual nests with one-phase embryos sometimes proved genetically to have multiple mothers, and, conversely, embryos of distinct developmental phases in a nest in some cases shared the same mother. In the latter case, presumably a mother spawns only a portion of her clutch in a male's nest and returns later to spawn again with the same male.

In summary, our genetic analyses of a tessellated darter population have confirmed several suspicions from behavioral studies in the field (Atz, 1940; Constantz, 1979, 1985). These include the fact that (1) most offspring are sired by the attendant male, and, thus, nest site fidelity is high; (2) by virtue of nest takeovers, guardian males sometimes defend nests containing offspring that they did not sire; (3) fertilization thievery may occur as well (albeit at low frequency in this study population); and (4) many females routinely spawn successfully in a single nest. This study illustrates how genetic analyses coupled with field observations can address otherwise clandestine elements in the spawning behavior and reproductive success of fishes.

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APPENDIX. MOLECULAR AND POPULATION ATTRIBUTES OF SIX MICROSATELLITE PRIMER PAIRS IN *Etheostoma olmstedi*. REACTIONS BEGAN WITH AN INITIAL TWO-MINUTE DENATURATION AT 94 C, FOLLOWED BY 32 CYCLES OF THE INDICATED PROFILE. THERMAL PROFILES CONSIST OF TIME (IN SECONDS) SPENT DENATUING AT 94 C, THEN ANNEALING AT THE INDICATED TEMPERATURE, AND THEN EXTENDING AT 72 C. HETEROZYGOSITIES ARE BASED ON A SAMPLE OF 36 PRESUMABLY UNRELATED ADULTS FROM THE FOURMILE CREEK STUDY SITE.

Primer name, fluorescent label, and sequence	Thermal profile	[Primer]	Avg. exp. het.	Avg. obs. het.
EO4F, 6FAM, 5′-CAGAGAAGATGTTTGCACTTC-3′ EO4R 5′-GTGAGGAGGGATAGCAGGC-3′	2, 2, 8 @ 55 C	0.25 μM	0.583	0.611
EO6F 5′-AACAGATGATGCTCAGTGG-3′ EO6R, HEX, 5′-ATCGACGACATACGAGTTCTG-3′	4, 4, 20 @ 57 C	0.33 μM	0.505	0.622
EO7F, HEX, 5′-ACTGTGCTGTTGAGAAATGC-3′ EO7R 5′-ACTGACCTTGTTTCAATGAG-3′	30, 30, 30 @ 48 C	0.33 μM	0.757	0.735
EO9F, NED, 5′-CCAGTGTGATAAGCTTGCCA-3′ EO9R 5′-CTGACAAGTGGCTGACATCA-3′	4, 4, 20 @ 51 C	0.17 μM	0.535	0.472
EO12F 5′-GTCAGATAGTCACTGCAACAG-3′ EO12R, 6FAM, 5′-CAGTAACGGCAGTCAGCAGAAG-3′	4, 4, 20 @ 57 C	0.25 μM	0.226	0.243
D1F 5′-CTCATCCATATTGCCTTGAGAGG-3′ D1B, NED, 5′-CTAACATTACATTGCTATTGAG-3′	4, 4, 20 @ 51 C	0.33 μM	0.622	0.694