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NEST MATERIALS AS A SOURCE OF GENETIC DATA FOR AVIAN ECOLOGICAL STUDIES

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Abstract.—We examined the utility of feathers and egg shell membranes, deposited in the nests of Spectacled Eiders (*Somateria fischeri*), as a source of DNA for genetic studies at both the population and individual level. The potential for feather DNA contamination as a result of female behavioral interactions (e.g. nest parasitism), reuse of nest sites from previous years, or other unknown occurrences was acknowledged and specifically tested. DNA was successfully extracted from both feathers and egg shell membranes and waterfowl microsatellite loci were used to construct individual genotypes. We found no difference in the genotypes obtained from nest feathers or blood of the incubating female. Detection of nest feather contamination was possible with as little as one feather when samples from multiple females were intentionally mixed. Triplicate DNA extractions from 33 nests provided a means of detecting contamination in 3 nests. Egg membranes proved a viable source of offspring DNA and can contribute valuable data to investigations of parentage when assayed jointly with maternal feather DNA. Nest materials provide an efficient, non-invasive method of genetic sampling that can be readily incorporated into field research. However, the natural history traits and mating strategies of a species must be considered during sample collection to identify the possible sources of nest materials (e.g., paternal, maternal, parasite, etc.). Specific experiments should also be designed to test sampling assumptions.

MATERIAL DEL NIDO COMO FUENTE PARA OBTENER DATOS GENÉTICOS EN ESTUDIOS ECOLÓGICOS

Sinopsis.—Estudiamos la utilidad de plumas y membranas de los huevos, depositados en el nido de individuos de *Somateria fischeri*, como fuente de ADN para estudios genéticos a nivel individual y poblacional. El potencial de contaminación del ADN como resultado de interacciones con hembras (ej. parasitismo reproductivo), utilización del nido en años previos, u otros factores fue reconocido y específicamente puesto a pruebas. Se extrajo ADN de plumas y de las membranas de los huevos y se utilizaron locis microsatelites de aves acuaticas para contruir genotipos individuales. No se encontraron diferencias en el genotipo obtenido de las plumas o la sangre de hembras que estaban incubando. Se detectó contaminación con tan solo una pluma cuando se mezclaron intensionalmente muestras de diferentes hembras. La extracción de ADN en triplicados de 33 nidos dio lugar a detectar contaminación en tres nidos. Las membranas de huevos probaron ser una fuente confiable del ADN de los pichones, que puede contribuir a investigaciones dirigidas a establecer relaciónes filiopaternales utilizando material genético encontrado en la pluma de adultos. El material encontrado en el nido provee de una forma eficiente y de un método no invasivo para obtener datos

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genéticos en estudios de campo. Sin embargo, la historia natural y las estrategias de apariamiento de la especie deben ser considerados durante la recolección de datos, para identificar las posibles fuentes de material en el nido (ej. paternal, maternal, la especie que parasíta, etc). Se deben diseñar experimentos para muestrear posibles escenarios o supuestos.

Molecular genetic techniques have enhanced our ability to draw ecological and evolutionary inferences from natural populations (Burke et al. 1992). Genetic data can help resolve issues of species evolution, gene flow, and mating behavior not detectable through direct observation (Avise 1996). In regards to behavior, genetic markers such as microsatellite loci (Bruford and Wayne 1993, Tautz 1989) are particularly insightful as they can uniquely identify an individual with probabilities exceeding 10^{-5} when multiple loci are used (Fields and Scribner 1997, Paetkau and Strobeck 1994). Additionally, alleles are inherited in a Mendelian fashion that permits rapid determination of parentage and estimation of genetic relatedness among individuals (Blouin et al. 1996, Westneat and Webster 1994). Microsatellite genotypes also exist in either the homozygous or heterozygous state rather than as a series of fragments where allelic states and specified loci are usually unknown (i.e., as in minisatellite DNA systems; see Scribner et al. 1994).

Standard protocols for genetic investigations of wild avian populations typically involve the collection or physical capture of a large number of individuals and blood or tissue sampling. Furthermore, examinations of mating behavior and reproductive success require sampling of putative parents and associated offspring. When properly performed, capture and blood sampling impose minimal survival consequences to wild birds (Hoysak and Weatherhead 1991, Lanctot 1994, Lessells et al. 1996). However, for studies involving sensitive species (e.g., threatened or endangered) or when disturbance associated with trapping and handling compromises project goals, traditional sampling methods may be unsatisfactory. For studies of parentage, sampling must be coincidental with hatching because precocial young are often cryptic and highly mobile (Lanctot 1994) and nest mortality of altricial species early in life can alter results.

An alternative to sampling birds directly is the use of DNA-containing materials deposited by birds in nests. Most species of waterfowl (Anatidae) deposit down and contour feathers in nests as incubation advances (Bellrose 1980) and egg shell membranes remain in the nests after hatch. Previously, DNA has been successfully extracted and analyzed from both plucked and naturally shed feathers (Ellegren 1992, Morin et al. 1994, Taberlet and Bouvet 1991). These authors suggested that non-invasive sampling of feathers for DNA fragments, coupled with the polymerase chain reaction (PCR; Saiki et al. 1988) to amplify minute and even highly degraded DNA (Arnheim et al. 1990), could eliminate the need to collect wild birds and greatly reduce disturbance and effort associated with trapping and blood or tissue sampling.

However, potential contamination problems exist in the sampling of nest materials as a result of a species' natural history. If both members of a breeding pair incubate the eggs, nest feathers may yield a mixture of

maternal and paternal DNA. Altricial young may contribute feathers during development to fledging. Feathers from other species may be incorporated into the nest lining during construction of the nest or deposited by interspecific brood parasites. Alloparental care or conspecific nest parasitism may also result in the presence of non-host feather and egg membrane DNA. Nest sites may be used over several breeding seasons, but by different individuals. Finally, feather and egg membrane DNA will be present in smaller quantities than obtained from blood samples and may be highly degraded by ambient environmental conditions.

We employed microsatellite loci, cloned from waterfowl species (Fields and Scribner 1997), to investigate the use of nest feathers and egg membranes as part of a study concerning female breeding philopatry and conspecific nest parasitism in the Spectacled Eider (*Somateria fischeri*). The Spectacled Eider is a threatened sea duck (U.S. Fish and Wildlife Service 1993) that nests in coastal tundra of arctic Alaska and Russia (Bellrose 1980, Stehn et al. 1993). Males depart breeding areas soon after egg laying and play no role in incubation or brood rearing. While no paternal feathers are believed to be deposited in nests, there is the potential for multiple females to contribute feathers to a single nest as conspecific brood parasitism has been documented in Common Eiders (*Somateria mollissima*; Bjørn and Erikstad 1994, Robertson et al. 1992) and is suspected in Spectacled Eiders as a result of genetic investigations of egg membranes (Pearce, unpubl. data).

Our desire to develop a non-invasive sampling protocol for genetic analysis that did not necessitate physical handling or disturbance, coupled with a concern for sample contamination as a potential result of Spectacled Eider natural history, warranted an examination of the suitability of nest materials as a DNA source. Specifically, does nest feather DNA provide the same genotype as that obtained from blood DNA collected via capture of the incubating female? Do multiple feather DNA extractions from a single nest provide consistent genotypes? If feathers from more than one female are present in a nest, how readily is such contamination detected? Finally, do egg membranes provide a reliable source of offspring DNA?

METHODS

Field sampling.—Weekly nest searches were conducted during 1993–1995 on two study areas, the Indigirka River Delta, Russia (71°20′N, 150°20′E) and Yukon-Kuskokwim Delta, Alaska (61°20′N, 165°35′W) after the peak of egg laying. Active (containing at least one egg) and depredated (nest bowls containing some or abundant down) nests were assigned unique numbers. Nest location, status, amount of feather material, and stage of egg development were recorded. Active nests were monitored every 7 d and eggs were numbered at both ends with a permanent marking pen. Once hatched, intact and broken egg shells were identified by number and stored separately to prevent cross-contamination. As only the vascularized chorioallantois membrane tissue is used in DNA extrac-

tions, the outer shell membrane was discarded in the field. Membranes were stored in 100% ethanol at room temperature until DNA extractions were conducted. However, we have extracted sufficient quantities of high molecular weight DNA from avian egg shell membranes stored dry in air tight containers for 3 yr. Following hatch or depredation of a nest, all available contour feathers were removed from down within the nest bowl and stored frozen in small plastic bags. Blood samples were collected by nest trapping incubating hens and placing approximately three drops of blood into 1 ml of blood storage buffer (100mM Tris pH 8.0, 100mM EDTA, 0.5% SDS, 10mM $NaCl_2$), which can be stored at ambient temperature for extended periods (Longmire et al. 1988). The field collection protocol presented here is ideal for eiders, but may need to be altered depending upon the species being studied and the logistical and personnel constraints of the particular research project. However, regardless of the sampling protocol employed, the collection and storage conditions must ensure that sample cross-contamination is prevented.

Feather DNA extractions.—While single feathers have been used previously (Ellegren 1992, Taberlet and Bouvet 1991), we used four feathers per extraction to maximize PCR product visibility and detect contamination, if present. Feathers were briefly soaked in 70% ethanol to prevent vane material from potentially contaminating the preparation area. Ethanol may also remove surface contamination from the feather, but this was not examined. The entire vane was stripped from the central rachis and each rachis was minced into 0.25 cm fragments with a razor blade and then placed into a 1.5 ml Eppendorf tube. Latex gloves were worn throughout the procedure and instruments were washed with ethyl alcohol between each sample preparation. Ideally, a separate sterile razor blade would be used for each sample. However, results below and from other studies involving feather DNA (Pearce 1996) do not suggest that sample cross-contamination is occurring during feather preparation.

DNA extractions followed a Chelex resin protocol (Morin et al. 1994). Feather fragments were transferred to a sterile 1.5 ml tube containing 0.25 ml of 5% Chelex and boiled for 45 min in a heat block. Every 15 min during boiling, samples were vortexed 10 s and centrifuged for 10 s. Following the final vortex, samples were centrifuged 2 min at 14,000 rpm and the supernatant removed and spun through filter tubes (Millipore 30,000 NMWL regenerated cellulose) with two washes of 0.5 ml sterile dH_2O. Extracted DNA was eluted from the filter in 100 μl of sterile dH_2O and 10 μl aliquots were used in each PCR reaction.

Blood and egg shell membrane DNA extractions.—In preparing blood sample DNA, approximately 200 μl of the blood and blood buffer solution mixture were transferred to 1.5 ml tubes and used in DNA extractions. For each individual egg membrane, a 3-cm piece of vascularized chorioallantois membrane was excised from the inner shell membrane and placed in a 1.5 ml tube. Instruments and working area were washed with ethyl alcohol between each membrane preparation. Both membrane and blood DNA was extracted using a standard proteinase K, phenol/chlo-

roform, and ethanol precipitation protocol (Sambrook et al. 1989). Extracted DNA was resuspended in TE (10mM Tris, pH 8.0, 1mM EDTA) and quantified by fluorimetry. We used 100 ng for each PCR amplification. Various extraction kits also produce sufficient quantities of DNA from both feathers and egg membranes, eliminating the use of caustic materials such as phenol and chloroform.

Microsatellite amplification and genotype determination.—We used PCR primers and conditions for the polymorphic waterfowl *Sfi*μ5 locus (Fields and Scribner 1997) in all genotype determinations. This locus was chosen due to the large number of alleles ($n > 20$) present in Spectacled Eiders and the high probability of identity (0.025; Pearce 1996). Only one locus was used in these experiments, but multiple microsatellite loci should be used when addressing questions of individual identity and parentage to decrease the probability of two birds having the same genotype by chance. PCR cocktails utilized 1 pmole of forward primer end-labeled with [$\gamma - {}^{32}$P] ATP using T4 polynucleotide kinase according to manufacturers' specifications (Pharmacia). Non-radioactive techniques are also available (see Sanguinetti et al. 1994). We used a 25 μl total volume containing template DNA, sterile dH_20, PCR buffer (1M Tris HCl pH 8.3, 1M $MgCl_2$, 1M KCl, 100 mg BSA, 0.025% Tween 20), 200 μmol dNTPs each, 10 pmole unlabeled reverse primer, 9 pmole unlabeled forward primer, and 0.25 units *Taq* polymerase enzyme. PCR was carried out in a Perkin Elmer thermal cycler using the following profile: 1 cycle at 94 C for 2 min followed by 40 cycles of 94 C for 1 min and 47 C for 1 min. No extension step was used. After PCR, 10 μl of stop solution (95% formamide, 0.05% xylene cyanol, 0.05% bromphenol blue) was added to each reaction. Microsatellite genotypes were resolved on a 6% denaturing polyacrylamide gel. An M13 control sequence (USB Sequenase kit) was run adjacent to PCR products on each gel as an unambiguous size standard. A PCR negative control mix (not containing DNA) was also used in each test to ensure absence of PCR contamination. Gels were dried and genotypes visualized by autoradiography.

Testing suitability of nest material DNA.—To test the assumption that DNA obtained from nest feathers can be used to identify the nesting female, microsatellite genotypes of five females sampled for both blood (obtained through physical trapping) and nest feathers were analyzed. Samples were loaded on separate polyacrylamide gels and scored independently by two laboratory personnel. To examine the detection probability of female contamination within a given feather extraction (i.e., the presence of feathers from two or more females in a single nest), we knowingly mixed various amounts of feathers from two females heterozygous for alternate alleles. As all extractions involve four feathers, mixtures contained 4 and 0, 3 and 1, 2 and 2, 1 and 3, and 0 and 4 feathers from the two females, respectively. Samples were run in a side-by-side comparison. PCR amplification of microsatellite loci results in one or two product bands on autoradiographs per individual, denoting homozygositiy or heterozygosity. Any additional bands are the result of sample contamination

or PCR artefact (see discussion). To test the assumption that four nest feathers from a single nest will yield the same genotype, triplicate extractions were performed on a random sample of 33 nests. First, an initial extraction was conducted from each sample and genotypes determined. Two additional extractions were then prepared from the same samples and run side-by-side on polyacrylamide gels. Genotypes from the second round of extractions were scored without knowledge of the original allele assignments. Genotypes from the three extractions were examined for consistency and presence of any contaminants. To examine if egg membranes provided a reliable source of offspring DNA, genotypes from four egg shell membranes were examined for the presence of a maternal allele present in feather DNA from the same nest.

RESULTS

Feather DNA.—In 1995 on the Indigirka River Delta, 90% of all nests discovered (n = 184) contained between 10 and 50 contour feathers suitable for DNA extraction. Most nests on the Indigirka River discovered during laying (containing 0–3 eggs) contained no feathers (73% in 1994, n = 37; 75% in 1995, n = 28), leading us to assume that nest feathers from previous years were not present and that down accumulated during incubation.

Comparisons of blood and feather samples from five nesting females revealed identical genotype designations in each case (data not shown). Intentional sample contamination with various amounts of feathers showed that contamination can be detected with as few as one feather from an additional female (Fig. 1). Also, PCR product intensity decreased when fewer feathers were used in an extraction. Of the 99 extractions conducted for the feather consistency experiment, only four extractions (4%) did not produce any visible PCR product (e.g., Fig 2., replicate 2 of sample 48). Most importantly, each replicate matched previous genotype designations (data not shown). However, three samples exhibited signs of possible contamination (Fig. 2, only sample 23 shown) as faint additional bands were present in one, but not all extractions.

Egg membrane DNA.—Extractions from the four membranes yielded 20–500 ng/μl of DNA. The large variation in DNA yield is most likely due to the amount of vascularization in the membrane sample and extraction efficiency. Additional membrane extractions that we conducted as part of other studies on the Yukon-Kuskokwim Delta, yielded an average of 228 ng/μl of DNA per sample (n = 17, range 120–400 ng/μl). Genotypes from the four egg membranes exhibited strict inheritance of one allele from the maternal genotype, amplified from nest feather DNA, and one allele from the unknown paternal genotype (Fig. 3), as is expected with microsatellite loci (Fields and Scribner 1997, Tautz 1989).

DISCUSSION

Through the experiments detailed above, we find that nest materials provide a practical source of DNA for examining behavioral and popu-

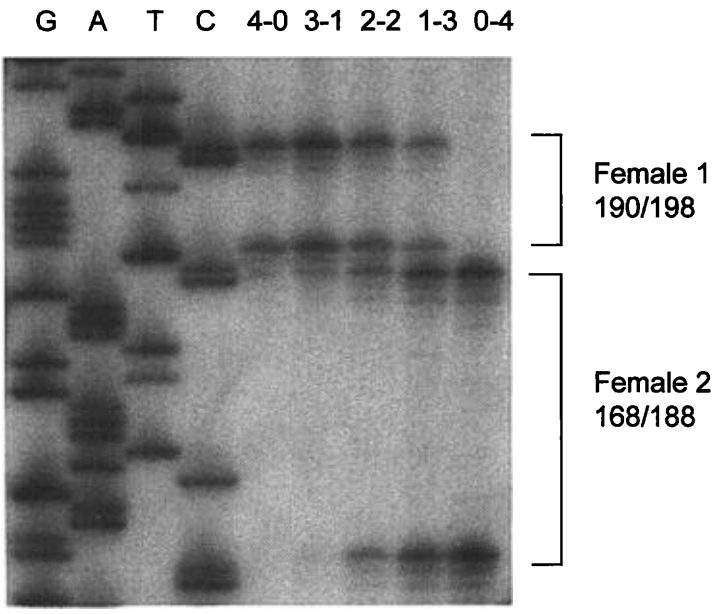

FIGURE 1. Autoradiograph of intentional contamination of feather samples. Numeric combinations correspond to the number of feathers from each female used per extraction. Genotype designations are given for each female (in base pairs) using the M13 sequencing ladder shown at left.

lation level questions. The use of nest materials as a source of DNA can also greatly reduce disturbance to nesting adults and potentially produce larger sample sizes than through trapping. By using a single microsatellite locus, we confirmed that nest feathers do provide the genotype of the incubating female. The presence of an additional female was detectable even with only one feather (Fig. 1). This result was also evident at other polymorphic waterfowl microsatellite loci (data not shown). Finally, parentage of putative offspring was preliminarily determined by comparing parental and offspring genotypes after extracting DNA from feathers and egg membranes from the same nest.

We used a single microsatellite locus to demonstrate the feasibility of nest materials as a DNA source. However, data presented here are preliminary and multiple polymorphic loci must be used in any question regarding the assignment of individual identity and parentage to decrease the probability that individuals share alleles by random chance. For example, the probability that two individuals share the same genotype decreases to near 10^{-5} when ≥ 4 loci are employed (Paetkau and Strobeck 1994, Pearce 1996).

While extraction tests conducted in this study confirmed our sampling assumptions that permits confident assignment of genotypes to individuals, potential problems also exist in the methodology of PCR amplified

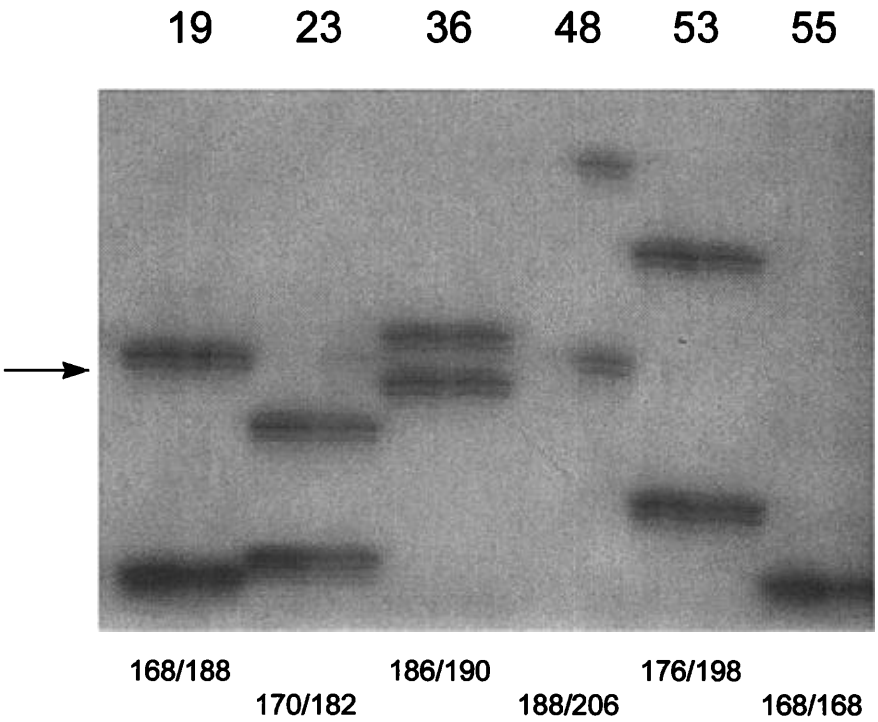

FIGURE 2. Consistency of feather genotypes from duplicate DNA extractions run side-by-side. The second extraction of sample 48 did not show a PCR product. The arrow at left denotes a faint third contaminant allele present only in the third extraction of sample #23. Genotype designations for each individual are given at the bottom of the figure.

microsatellite loci. Erroneous genotype scoring can occur if a shorter, contaminant allele out competes larger, host alleles during PCR amplification, resulting in a more intense short allele product due to the decreased replication time (Hõss et al. 1994). PCR primers can also produce artefact bands within the range of allele sizes at a particular locus due to imperfect homologies between primer and template (Don et al. 1991), through *Taq* polymerase slippage that creates stutter bands (Ellegren 1992, Tautz 1989), or as the result of primer-primer structures, especially when allele products are <100 base pairs. In the above situations, additional purification of DNA templates, PCR optimization experiments (see Don et al. 1991), or the redesign of primer sequences can improve product clarity. Finally, the occurrence of null alleles at some microsatellite loci (Pemberton et al. 1995) can result in inaccurate allele assignments. Due to mutations in a priming site, null alleles are not replicated during PCR and samples appear either homozygous or lacking PCR product. The potential for laboratory contamination must also be constantly monitored. Negative controls should always be used in PCR reaction experi-

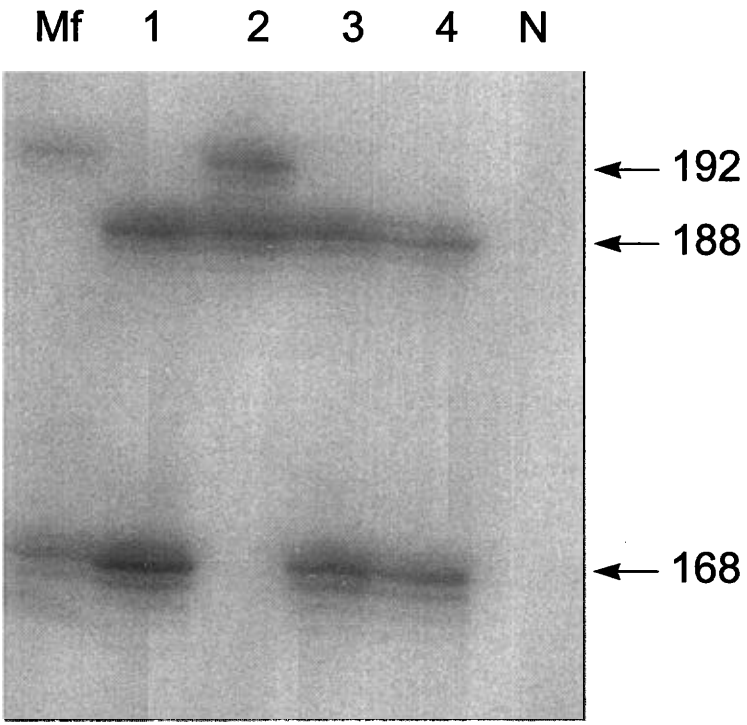

FIGURE 3. Mendelian inheritance of microsatellite alleles amplified from egg membranes. Mf corresponds to the maternal genotype from feather DNA. Offspring are numbered 1–4. An M13 sequencing ladder is shown at left. N corresponds to the PCR negative control. Individual alleles are labeled at right.

ments to ensure absence of foreign DNA in reaction chemicals and buffers. Negative controls can also monitor the purity of solutions used in DNA extractions.

In conclusion, the applicability of nest feathers as a DNA source extends beyond the waterfowl as other bird species, such as swallows (Møller 1987, Winkler 1993), grebes, shorebirds, galliformes, raptors (Ehrlich et al. 1988), and some passerines (Møller 1984) use feathers in nest construction and incubation. However, the specific nesting characteristics of each species (i.e. breeding behavior, nest construction, altricial vs. precocial young) must be considered and potential contamination sources identified and tested. Once sampling assumptions are confirmed, the detection of contamination with a molecular tool such as microsatellite loci, may allow inferences to be made regarding intraspecific behavioral processes that occur during the nesting period. Microsatellite loci also have the potential to identify interspecific interactions that may occur at a nest site as PCR allele products often have species specific size ranges (e.g. Primmer et al. 1996), allowing species identification.

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