



Eggs yield nuclear DNA from egg-laying female cowbirds, their embryos and offspring

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Abstract

Here we report methods for extracting maternal DNA from avian eggshells or offspring DNA from eggshells and embryos. These methods offer alternative techniques for obtaining DNA from oviparous organisms. Using DNA extracted from eggshells, we obtain microsatellite genotypes of the brood parasitic brown-headed cowbird (*Molothrus ater*) female that laid the eggs and/or her hatched offspring. Using DNA extracted from embryos, we obtain microsatellite genotypes of offspring. We demonstrate that separate extractions performed on the embryo and shell from a single egg can provide DNA from the embryo and its mother, respectively. This single-egg approach for obtaining both maternal and embryonic DNA simplifies paternity analyses because alleles unique to the embryo can be considered paternal in origin. Finally we report two new microsatellite loci and primer sequences for brown-headed cowbirds.

Introduction

Genetic analysis has provided important new insights into the ecology and conservation of many bird species, particularly with regards to kinship and mating systems (e.g. Westneat and Webster 1994; Dunn and Lifjeld 1994). Acquiring samples for genetic analysis of wild birds is often difficult, and is particularly challenging for species that lay eggs in the nests of other birds of the same or different species; strategies known as intraspecific and interspecific brood parasitism, respectively (Westneat et al. 1987; see also Strausberger and Ashley 1997). Researchers have no *a priori* information on potential mothers and mothers are often very inconspicuous and may never return to the nest or nest area following laying (Fitch and Shugart 1984).

Invasive sampling methods for birds have yielded DNA from blood and plucked feathers (e.g., Taberlet and Bouvet 1991; Petitte et al. 1994). Noninvasive sources for bird DNA include molted feathers (Leeton and Christids 1993; Taberlet et al. 1999) and the vascularized chorioallantoic membrane from

the inside of post-hatch eggs (Pearce et al. 1997). Although not previously demonstrated, eggshells may also be a source for maternal DNA. It is unlikely that the shells of newly laid eggs or eggs in early developmental stages will be contaminated by embryonic DNA because, when laid, the embryonic blastoderm within an egg is small and limited to a discrete area away from the shell (Romanoff and Romanoff 1949). The embryonic tissue rests largely on upright surface of the yolk and is separated from the shell, and its membranes, by albumen. It is not until after incubation begins that the developing embryo's highly vascularized chorioallantoic membrane attaches to the shell's inner membrane (Glaser and Piehler 1934).

Here, we demonstrate for the first time extraction of maternal DNA from avian eggshells. We use new extraction methods to acquire DNA from naturally laid eggs of the interspecific brood parasitic brown-headed cowbird (*Molothrus ater*). Using DNA extracted from eggshells, we obtain microsatellite genotypes for the females who laid the eggs and/or their offspring. We also demonstrate successful extraction of offspring DNA from cowbird eggshells

and embryos. Finally, we expand on Pearce et al.'s (1997) method by showing that offspring DNA can be obtained directly from post-hatch eggshells without removing associated membranes.

Methods

Cowbird eggs and offspring were sampled from a population at the Morton Arboretum in Northeastern Illinois (see Strausberger and Ashley 1997; Strausberger 1998a). To determine the length of time required for the shell and associated membranes to acquire embryonic DNA, eggs were incubated either 1–2 days, 2–4 days, or until the egg hatched (see Strausberger 1998b). Cowbird eggs have an incubation period of 11.6 days (Strausberger 1998b). For egg dissections, we used disposable gloves, weigh boats, razor blades, and pipette tips and sterile forceps. All samples were placed into 1.5 ml microcentrifuge tubes and stored at -70°C for later DNA extraction.

Tissue collection and DNA extraction

Both maternal and embryo DNA extractions from a single egg

We used seven eggs incubated 1–2 days, and to reduce the possibility of shells being contaminated by host DNA, we only used eggs that had not been incubated in the nest. These included eggs laid in abandoned nests. We used three eggs incubated 2–4 days, and because eggs were limited, we used eggs that received partial incubation by hosts. To separate the maternal-shell and embryo samples of individual eggs, we placed eggs in disposable plastic weigh boats and used razor blades and forceps to remove and discard the upper one-third of shell opposite to where the egg contacted the weigh boat. Egg contents were then poured into a new weigh boat where the disc-shaped embryo (blastoderm) and associated extra-embryonic tissue were removed using a pipette. To reduce the possibility of the embryo contaminating the egg shells as the egg contents were poured out, we collected only the lower half of the shell for extraction (the part contacting the weigh boat). The remaining empty shell and egg contents were discarded.

Offspring samples from post-hatch eggshells or blood

To confirm that DNA from offspring could be obtained from post-hatch eggshells in their entirety,

we compared genotypes obtained from five post-hatch eggshells and associated membranes to individual offspring that had hatched from the eggs. Two fresh and three eggs that received partial incubation by hosts were used for comparing DNA from post-hatch eggshells and their respective hatchlings.

DNA extraction

DNA was extracted using standard phenol-chloroform extraction from approximately 1–6 μl of blood, aqueous embryonic fluid and associated tissue (Sambrook et al. 1989). DNA extracted from embryos was resuspended in 25 μl of water, whereas DNA extracted from blood was resuspended in 500 μl of water.

For eggshells, approximately 50 mg of shell and associated membranes was placed into a 1.5 ml microfuge tube and finely crushed with a toothpick. We added 0.7 ml of STE, 50 μl 10% SDS, and 10 μl of 10 mg/ml proteinase K to the shell sample. Following capping and sealing with parafilm, the samples were placed on a rocker for 6–12 hours at 55°C . Occasionally, samples were shaken aggressively to resuspend settled matter and placed back on the rocker. Following the digest, 0.7 ml of phenol-chloroform was added and the tube was vortexed for 10 sec following capping and sealing with parafilm. Tubes were then centrifuged for 10 min. The top layer was removed and placed into a new 1.5 ml tube and phenol-chloroform treated a second time, after which the DNA was precipitated. DNA extracted from post-hatched shells was resuspended in 100 μl of water, whereas DNA from pre-hatching eggshells was resuspended in 15 μl of water. DNA was quantified using a DyNA Quant 200 fluorometer.

Microsatellite detection, isolation, and primer construction

A microsatellite genomic library was constructed from brown-headed cowbird DNA using the general protocol of Rassmann et al. (1991) as modified by Dow et al. (1995). We digested genomic DNA with *Sau3I* and fragments were size fractionated on 1% agarose gels. pGEM plasmid DNA was digested with *BamHI* and dephosphorated with shrimp alkaline phosphatase (SAP, USB). Approximately 50 ng of insert *E. coli* DNA was ligated into 200 ng pGEM and incubated for 10 hours at 15°C , and transformed into competent cells (JM109). Bacteria colonies were plated, lifted onto nylon membranes, and screened for

microsatellite repeats with Amersham ECL-3' oligolabeling and detection system. Plasmid DNA from positive colonies was sequenced using Sequenase 2.0 (USB) and separated on 6% standard denaturing acrylamide gels. We designed fluorescently labeled primers using MacVector software (IBI) for loci *Maμ* 101 and *Maμ* 104. Primers sequences and allele size ranges are given in Table 1. In addition, primers *Maμ* 25 and *Maμ* 29 from Alderson et al. (1999) were used.

Microsatellite genotyping

PCR amplifications were carried out in a final volume of 7.5 μ l and contained 4–100 ng genomic DNA, 0.25 mM dNTPs, 0.2 μ M each primer, 0.17 μ g BSA, 1X PCR buffer, 0.3 U Amplitaq Gold Polymerase (PE Biosystems), and various amounts of MgCl₂ (5 mM for *Maμ* 104 and 25 or 2.5 mM for *Maμ* 101 and 29). To activate the polymerase, each PCR began with a hot start at 95 °C for 12 minutes and continued as follows. For *Maμ* 104, 25, and 29 there were 20 cycles of 94 °C for 5 seconds, 58 °C (annealing temperature) for 15 seconds, and 72 °C for 45 seconds followed by 20 cycles of 89 °C for 15 seconds, 58 °C (annealing temperature) for 15 seconds, and 72 °C for 45 seconds. For *Maμ* 101 the annealing temperature was reduced to 55 °C. PCR amplifications were conducted in a PTC-100 thermocycler (MJ Research Inc.). Because unhatched shell samples generally yielded small quantities of DNA (PCR reactions sometimes contained only 4 ng of genomic DNA), we repeated PCR reactions on unhatched shell samples from 2–6 times to confirm genotypes, depending on the quality of the resulting electropherograms. For allele scoring, a portion of PCR products (0.5 μ l) from each locus was combined with 3.5 μ l of formamide loading dye that contained the fluorescent labeled Tamara-350 size standard (PE Biosystems) and assayed using an ABI 373A automated sequencer. We used Genescan software to create electropherograms and to score PCR products.

Results and discussion

Our results indicate that a single egg can provide both maternal and offspring DNA. From seven eggs artificially incubated 1–2 days, we obtained 11–115 and 1–6 ng/ μ l DNA from embryo and shell samples, respectively, and we obtained genotypes for three or four loci for each sample (Table 2). We failed to obtain high quality electropherograms from one shell

sample for unknown reasons, indicating that not all shell samples may yield results. Occasionally, we were unable to amplify loci from DNA obtained from shells, perhaps due to low DNA yields. Our results suggest that DNA in eggshells was that of the egg-laying female because we obtained genotypes from the shell and embryo samples that were consistent with Mendelian mother-offspring relationships.

Because obtaining maternal DNA using this method requires destruction of eggs it may not be an option for some researchers. However, for many birds such as indeterminate layers who lay extra eggs to replace those that are removed, egg collection has minimal impact on their reproductive success (Haywood 1993; Gill 1995). A further advantage of using eggshells as a source for maternal DNA is that one is able to genotype egg-laying females without ever encountering them. A single-egg method for obtaining both maternal and embryonic DNA also greatly aids paternity assignment because alleles unique to the embryo can be considered paternal in origin. In addition to bird eggs, this method may be useful for other oviparous organisms.

From three eggs artificially incubated 2–4 days we obtained 290–813 ng/ μ l and 2–24 ng/ μ l DNA from embryo and shell samples, respectively. Results from eggs incubated 2–4 days suggest that embryos' DNA likely contaminated the maternal DNA present in the shell. The genotype data shown in Table 2 suggests that the DNA obtained from shells is not solely maternal in origin. For many loci, we obtained three alleles, two of which were present in the embryo; a pattern consistent with contamination by DNA from the embryo. For shells 594B and 599B we obtained three alleles each at loci *Maμ* 29 and 101, only one of which was shared by their respective embryo. Because one of these alleles cannot be from the mother/embryo combination it must represent an unknown artifact or contamination, possibly from hosts. The incubation period of cowbirds is possibly the shortest of any bird (see Ricklefs and Stark 1998; Strausberger 1998b). As a result, the shells of eggs from any species incubated for under two days are unlikely to be contaminated by embryonic DNA.

Contamination of the maternal shell sample after just 2–4 days of incubation suggests that this method will have little application for obtaining maternal DNA from incubated eggs found in nests beyond the egg laying stage. Nonetheless, many eggs are found in nests in this stage because incubation often does not begin until the clutch is complete or nearly so

Table 1. Characteristics of two microsatellite loci used for genotyping brown-headed cowbirds. See Alderson et al. (1999) for information regarding *Maμ* 25 and *Maμ* 29

Locus	PCR product length (bp) ¹	Repeat motif	Primer sequence (5'-3')	Range of allele sizes (bp)
<i>Maμ</i> 101	156	(AC) 15	F-GTTCATCTTCCTTCCTCTGCCTG R-TCCCTCTTCTGCTTTTGCTTTG	147–189
<i>Maμ</i> 104	144	(AC) 15	F-CTGAGGACCACCTTTGGGTTTC R-TGAGCAGCCATCCAGTTAATTC	137–185

¹Represents size of cloned and sequenced allele.

Table 2. Comparison of microsatellite allele sizes obtained from DNA extracted from either the embryo, hatching, or the shell from individual brown-headed cowbird eggs

Egg number	Source	<i>Maμ</i> 25 ¹	<i>Maμ</i> 29 ¹	<i>Maμ</i> 101	<i>Maμ</i> 104
Incubated ² 1–2 d					
593C	Embryo	129/133	138/146	155/155	153/153
	Shell	127/133	146/162	151/155	153/155
594A	Embryo	153/173	154/176	149/155	145/167
	Shell	(–) ³	136/176	149/151	145/145
594C	Embryo	145/153	154/160	149/151	151/155
	Shell	129/153	134/154	149/157	149/155
5910A	Embryo	129/151	136/164	153/155	149/175
	Shell	129/151	(–) ³	155/179	149/157
5111A	Embryo	129/133	168/170	149/189	155/155
	Shell	129/189	136/168	149/185	155/171
5112A	Embryo	129/157	134/176	149/155	155/169
	Shell	137/157	134/176	149/149	157/169
Incubated ⁴ 2–4 d					
594B	Embryo	135/173	154/176	149/155	145/177
	Shell	(–) ³	136/142/176	149/155/183	145/149/177
599B	Embryo	161/165	162/176	153/155	153/159
	Shell	127/161	142/176	149/155/183	149/159
677A	Embryo	127/147	136/144	151/155	143/145
	Shell	127/133	136/144/174	151/155/159	143/149
Hatched					
5233A	Hatchling	129/151	136/140	149/155	145/161
	Shell	129/151	136/140	149/155	145/161
5254B	Hatchling	163/163	140/154	153/175	163/173
	Shell	163/163	140/154	153/175	163/173
52616A	Hatchling	133/173	154/154	151/153	161/177
	Shell	133/173	154/154	151/153	161/177
52617A	Hatchling	127/137	138/178	153/155	149/155
	Shell	127/137	138/178	153/155	149/155
6911A	Hatchling	165/173	142/176	155/183	167/175
	Shell	165/173	142/176	155/183	167/175

¹Primer sequences obtained from Alderson et al. (1999).

²Eggs incubated artificially for 1–2 days were collected when fresh and did not contact host birds.

³No PCR product obtained.

⁴Eggs incubated 2–4 days received partial incubation from hosts.

(Gill 1995). The utility of this method for obtaining maternal DNA is aided by the fact that embryonic development ceases below 25–27 °C (Drent 1975). Thus, fresh eggs kept below this temperature will allow for the extraction of maternal DNA many days after collection. Additionally, fresh eggs can remain viable and developmentally unchanged for days or even weeks (Romanoff and Romanoff 1949). For eggs with an unknown incubation history, candling (examining of an egg's content by placing it between one's eye and a light source; see Lokemoen and Koford (1996)), would prevent the collection of advanced stage eggs that are unsuitable for maternal DNA collection. The contamination of egg shells with offspring DNA during embryonic development could be avoided by reducing, or even eliminating, the incubation period. Fresh laid, fertile eggs likely contain sufficient offspring DNA for PCR based studies. For example, the blastoderm of a fresh laid chicken egg contains up to 60,000 nucleated cells (Burley and Vadehra 1989).

The shell and associated membranes from post-hatch eggs can be used as a source of offspring DNA. This procedure will simplify the method described by Pearce et al. (1997) by eliminating the need to remove the shells inner membranes. This will be especially useful for very small eggs where the shells are delicate and their inner membranes are particularly difficult to extract. Post-hatch eggshells and blood samples from five offspring yielded 29–110 and 210–714 ng/ml of DNA, respectively. We confirmed that the DNA obtained from post-hatch shells matched that of the hatchling from the egg by comparing the genotypes obtained from each (Table 2). The high quantity of DNA from post-hatch shells, compared to fresh shells, is likely attributed to the fact that avian blood cells are nucleated and yield high amounts of DNA (Rowley and Ratcliffe 1988; Seutin et al. 1991) and because blood residue was present in the attached shell membranes. The high quantities of offspring DNA relative to maternal DNA in post-hatch shells likely preclude detection of maternal alleles or those from incubating hosts.

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References

- Alderson GW, Gibbs HL, Sealy SG (1999) Parentage and kinship analyses in an obligate brood parasitic bird, the brown-headed cowbird (*Molothrus ater*) using microsatellite DNA markers. *J. Hered.*, **90**, 182–190.
- Dow BD, Ashley MV, Howe HF (1995) Characterization of highly variable (GA/CT)_n microsatellites in the bur oak, *Quercus macrocarpa*. *Theor. Appl. Genetics*, **91**, 137–141.
- Drent R (1975) Incubation. In: *Avian Biology*, vol. 5 (eds. Farner DS, Kink JR, Parkes KC), pp. 333–420. Academic Press, New York.
- Dunn P, Lifjeld J (1994) Can extra-pair copulations be used to predict extra-pair paternity in bird? *Anim. Behav.*, **47**, 983–985.
- Fitch MA, Shugart GW (1984) Requirements for a mixed reproductive strategy in avian species. *Am. Nat.*, **124**, 116–126.
- Flagstad Ø, Røed K, Stacy JE, Jakobsen KS (1999) Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. *Mol. Ecol.*, **8**, 879–883.
- Gill FB (1995) *Ornithology*, 2nd edn. WH Freeman and Company, New York.
- Glaser O, Piehler E (1934) The mobilization of calcium during development. *Biol. Bull.*, **66**, 351–356.
- Haywood S (1993) Sensory and hormonal control of clutch size in birds. *Q. Rev. Biol.*, **68**, 33–60.
- Leeton P, Christidis L (1993) Feathers from museum bird skins – a good source of DNA for phylogenetic studies. *Condor*, **95**, 465–466.
- Lokemoen JT, Koford RR (1996) Using candlers to determine the incubation stage of passerine eggs. *J. Field Ornithol.*, **67**, 660–668.
- Lowther PE (1993) Brown-headed cowbird (*Molothrus ater*) no. 47. In: *The Birds of North America* (eds. Poole A, Gill FB), pp. 1–24. Academy of Natural Sciences, Philadelphia and the American Ornithologists' Union, Washington, D.C.
- Pearce JM, Fields RL, Scribner KT (1997) Nest materials as a source of genetic data for avian ecological studies. *J. Field Ornithol.*, **68**, 471–481.
- Petitte JN, Kegelmeyer AE, Kulik MJ (1994) Isolation of genomic DNA from avian whole blood. *BioTechniques*, **17**, 664–666.
- Rassmann K, Schlötterer C, Tautz D (1991) Isolation of simple-sequence loci for use in polymerase chain reaction-based DNA fingerprinting. *Electrophoresis*, **12**, 113–118.
- Ricklefs RE, Stark JM (1998) Embryonic growth and development. In: *Avian Growth and Development* (eds. Stark JM, Ricklefs RE), pp. 31–55. Oxford University Press, New York.
- Romanoff AL, Romanoff AJ (1949) *The Avian Egg*. John Wiley and Sons, Inc. New York.
- Rowley AF, Ratcliffe NA (1988) *Vertebrate Blood Cells*. Cambridge University Press, Cambridge, New York.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

- Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Can. J. Zool.*, **69**, 82–90.
- Strausberger BM, Ashley MV (1997) Community-wide patterns of parasitism of a host “generalist” brood-parasitic cowbird. *Oecologia*, **112**, 254–262.
- Strausberger BM (1998a) Temporal patterns of host availability, brown-headed cowbird brood parasitism, and parasite egg mass. *Oecologia*, **116**, 267–274.
- Strausberger BM (1998b) Temperature, egg mass, and incubation time: a comparison of brown-headed cowbirds and red-winged blackbirds. *Auk*, **115**, 843–850.
- Taberlet P, Bouvet J (1991) A single plucked feather as a source of DNA for bird genetic studies. *Auk*, **108**, 959–960.
- Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: Look before you leap. *Trends Eco. Evol.*, **14**, 323–327.
- Westneat DF, Frederick PC, Wiley RH (1987) The use of genetic markers to estimate the frequency of successful alternative reproductive tactics. *Behav. Ecol. Sociobiol.*, **21**, 35–45.
- Westneat DF, Webster MS (1994) Molecular analysis of kinship in birds: Interesting questions and useful techniques. In: *Molecular Ecology and Evolution: Approaches and Applications* (eds. Schierwater B, Strait B, Wagner GP, DeSalle R), pp. 91–126. Birkhäuser Verlag, Basel, Switzerland.