Tools and Technology Note

Diagnostic Genetic Marker That Differentiates Eastern Fox Squirrels From Eastern Gray Squirrels

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ABSTRACT The Delmarva fox squirrel (*Sciurus niger cinereus*) has been listed as endangered by the United States Department of Interior since 1967. A high-priority task for the recovery of this taxon is to determine its current geographic distribution. Toward this end, we have identified a microsatellite locus that unambiguously differentiates Delmarva fox squirrels from eastern gray squirrels (*S. carolinensis*), which frequently co-occur with Delmarva fox squirrels. Analysis of this marker in noninvasively collected hair samples will allow unequivocal identification of localities occupied by Delmarva fox squirrels with a minimum investment of funds, time, and effort because handling individuals will be unnecessary. This protocol will expedite site review in connection with the Endangered Species Act consultation process. (JOURNAL OF WILDLIFE MANAGEMENT 72(1):320–323; 2008)

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The eastern fox squirrel (*Sciurus niger*) occurs throughout the eastern United States, from New York west to North Dakota and Manitoba, Canada, and south to Florida, Texas, and Coahuila, Mexico (Hall 1981). Ten subspecies are currently recognized (Hall 1981). Declining populations of this species have been documented for several subspecies, including the big cypress fox squirrel (*S. n. avicennia*), Delmarva fox squirrel (*S. n. cinereus*), southeastern fox squirrel (*S. n. niger*), and Sherman's fox squirrel (*S. n. shermani*; Loeb and Moncrief 1993). Of these, the Delmarva fox squirrel has been listed as endangered by the United States Department of Interior since 1967. It is also listed as a conservation-dependent species by the International Union for the Conservation of Nature and Natural Resources, the World Conservation Union (Nowak 1999).

Historically, the Delmarva fox squirrel occupied mature forests in Delaware and the Eastern Shore regions of Maryland and Virginia, as well as in southeastern Pennsylvania and western New Jersey, USA (Taylor 1973, Taylor and Flyger 1974, Lustig and Flyger 1976). Habitat loss caused by overcutting of mature forests, conversion of forestland to agriculture, and land development has reduced the distributional range of this taxon by approximately 90% (U.S. Fish and Wildlife Service [USFWS] 1993). The Delmarva fox squirrel is believed to persist naturally only in portions of Queen Anne's, Talbot, Caroline, and Dorchester counties in Maryland and Sussex County, Delaware (USFWS 1993, 2003). The USFWS (2003) deemed reintroduction attempts via translocation of animals to be successful at 11 sites in Kent, Somerset, Worcester, and Wicomico counties in Maryland, as well as in Accomack County, Virginia.

The distributional range of the Delmarva fox squirrel currently is disjunct from other subspecies of the eastern fox squirrel. In terms of geography, the 2 subspecies closest to the Delmarva fox squirrel are the eastern fox squirrel (*S. n. vulpinus*), which inhabits western Virginia, western Maryland, and Pennsylvania, and the southeastern fox squirrel, which occurs in southern Virginia, North Carolina, South Carolina, Georgia, and western Florida (Hall 1981).

A high-priority task for the recovery of the Delmarva fox squirrel is to determine its current geographic distribution within its historic range (USFWS 1993, 2003). However, this task is complicated by the fact that the eastern gray squirrel (*S. carolinensis*), which has similar ecological requirements, food habits, outward appearance, and leaf nest construction, occurs sympatrically, and often syntopically, with the Delmarva fox squirrel.

Traditional methods of unequivocally confirming the presence of Delmarva fox squirrels at a particular location have relied upon time-consuming, labor-intensive, and potentially disruptive methods such as live-trapping and nest box inspections. Therefore, we sought to develop a noninvasive method (analysis of DNA from plucked hair samples) for unambiguously detecting the presence of the Delmarva fox squirrel.

STUDY AREA

We collected hair samples from eastern fox squirrels and eastern gray squirrels at 3 localities within the range of the Delmarva fox squirrel (Patuxent Wildlife Research Center, Anne Arundel County, MD; Chincoteague National Wildlife Refuge, Accomack County, VA; Blackwater National

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Table 1. Number and type of samples[a] from known individuals of eastern gray squirrels and eastern fox squirrels captured between 2 February 1988 and 30 April 2002 in Allegany and Dorchester counties in Maryland, and in Alleghany, Augusta, Henry, Roanoke, and Sussex counties in Virginia, USA.

	Eastern gray squirrel		Eastern fox squirrel	
Locality	Liver	Hair	Liver	Hair
Allegany County, MD	10	0	9	0
Dorchester County, MD	9	18	10	31
Alleghany County, VA	3	0	4	0
Augusta County, VA	3	0	0	0
Henry County, VA	9	3	0	0
Roanoke County, VA	0	1	0	0
Sussex County, VA	0	0	2	0
No. of individuals	34	22	25	31
Total for each species	56		56	

[a] We obtained each sample from a different animal ($n = 112$).

Wildlife Refuge, Dorchester County, MD). We compared these to hair and liver samples (Table 1) from known individuals of eastern fox squirrels and eastern gray squirrels collected at 2 localities in Maryland (Allegany and Dorchester counties) and 5 localities in Virginia (Alleghany, Augusta, Henry, Roanoke, and Sussex counties).

METHODS

To ensure sufficient quantities of high-quality DNA for developing analytical protocols, we initially used liver samples of eastern gray squirrels and Delmarva fox squirrels to test 10 ground squirrel (*Spermophilus brunneus brunneus*) microsatellite loci (May et al. 1997). We then used one locus (IGS-110b) to analyze 112 liver and hair samples from eastern fox squirrels and eastern gray squirrels (Table 1).

We isolated DNA from liver samples using standard protocols (Longmire et al. 1997), and we isolated DNA from hair samples using the Chelex method (Walsh et al. 1991). For DNA extracted from liver, we carried out polymerase chain reaction (PCR) reactions in 15-μL reactions using 1.2 μL of purified DNA (not quantified), 0.5 μL each of 10-μM forward and reverse IGS-110b primers, 9 μL of True Allele PCR premix (Perkin-Elmer Applied Biosystems, Foster City, CA), and 3.8 μL of double-distilled H_2O (ddH_2O). We carried out PCR amplification in either an MJ Research PTC-100 (MJ Research Inc., Waltham, MA) or a Perkin-Elmer GeneAmp 9600 (Perkin-Elmer Applied Biosystems): 12 minutes at 95° C followed by 10 cycles of denaturation at 94° C for 15 seconds, annealing at temperatures that ranged from 43° C to 53° C for 1 minute, and extension at 72° C for 30 seconds; followed by 25 cycles of denaturation at 89° C for 15 seconds, annealing at 55° C for 1 minute, and extension at 72° C for 30 seconds, with a final extension step at 72° C for 30 minutes. For DNA extracted from hair, we used 6 μL of genomic DNA extract (not quantified), 1.5 μL of 25-mM $MgCl_2$, 0.3 μL each of 10-μM forward and reverse IGS-110b primers, 0.5 μL of 10-μM deoxynucleotide triphosphates, 4.8 μL of ddH_2O, 0.1 μL of Ampli*Taq* Gold polymerase (Perkin-Elmer Applied Biosystems), and 1.5 μL of 10× buffer supplied by Perkin-Elmer Applied Biosystems. We carried out PCR amplification in either an MJ Research PTC-100 or a Perkin-Elmer GeneAmp 9600: 10 minutes at 95° C; followed by 1 cycle of denaturation at 94° C for 30 seconds, annealing at temperatures that ranged from 43° C to 53° C for 30 seconds, and extension at 72° C for 30 seconds; followed by 39 cycles of denaturation at 94° C for 30 seconds, annealing at temperatures that ranged from 43° C to 53° C for 30 seconds, and extension at 72° C for 30 seconds; with a final extension step at 72° C for 2 minutes. For all extracts, we added 1.5 μL of PCR reaction to 3.5 μL of loading buffer containing 0.5 μL of GS-400HD ROX size standard (Perkin-Elmer Applied Biosystems), 0.5 μL of loading dye, and 2.5 μL of formamide. We denatured this mixture at 95° C for 5 minutes, then ran it through a 6% Long Ranger acrylamide gel (Cambrex BioScience, Rockland, ME) on a Perkin-Elmer ABI Prism 377 DNA Sequencer. We analyzed the resulting data using GENESCAN™ version 2.1 and GENOTYPER™ version 2.4 software packages (Perkin-Elmer Applied Biosystems).

After optimizing the protocols with liver samples, we used the IGS-110b locus (May et al. 1997) to analyze hair samples collected from 53 known individuals (31 Delmarva fox squirrels and 22 eastern gray squirrels; Table 1). We also conducted trials of different ratios of Delmarva fox squirrel and eastern gray squirrel hairs (1:19, 5:15, 10:10, 15:5, 19:1) to determine the minimum number of Delmarva fox squirrel hairs necessary, in a sample of 20 hairs, to detect Delmarva fox squirrel DNA. In all, we analyzed 41 samples of Delmarva fox squirrels and 27 samples of eastern gray squirrels from Dorchester County, Maryland (Table 1). In order to ensure that our results were valid without being confounded by intraspecific polymorphisms, we analyzed samples from 29 additional known eastern gray squirrels and 15 additional known eastern fox squirrels (Table 1). The eastern gray squirrels came from localities in western Maryland, western Virginia, and south-central Virginia. The eastern fox squirrels came from western Maryland and western Virginia and the southeastern fox squirrels came from southeastern Virginia.

Finally, we conducted a trial in April 2004 to test our field and lab techniques. We collected 23 samples (each of which consisted of 20 squirrel hairs from unknown individuals), using traps deployed at 3 different localities. We obtained 6 samples from Patuxent Wildlife Research Center, Anne Arundel County, Maryland, where only eastern gray squirrels were present. Nine samples were from Chincoteague National Wildlife Refuge, Accomack County, Virginia, where only Delmarva fox squirrels were present. Eight samples were from Blackwater National Wildlife Refuge, Dorchester County, Maryland, where both species were present.

One of us (R. D. Dueser [RDD]) assigned unique numbers to the traps and corresponded with field assistants who deployed all the traps at the 3 locations. After deployment, all the traps were returned to RDD, who labeled the samples with unique numbers. This procedure

ensured that none of the other coauthors, who were only involved in the lab phase of this study, knew the locations from which we collected the samples.

The hair traps consisted of a piece of white class C polyvinyl chloride (PVC) tubing fitted with 2 glue strips for collecting hair, a small internal bait container, and 3 steel rods to anchor the trap firmly to the ground. Each PVC tube was 10 cm in diameter and 60 cm in length. We firmly attached one glue strip to the inside top at each end of the tubing with carpenter's adhesive, with the outer edge of the strip inset approximately 1 cm inside the end of the tube.

The bait container was a piece of 7-mm wire mesh rolled into a 5-cm diameter cylinder just long enough to slide inside the tube, with the open ends against the top and bottom of the tube. We filled this container with cracked pecans, peanuts, and oily walnut meats. We then pinned the container with a vertical threaded steel rod that extended from the outside top surface of the tube, through the container, and out through the bottom of the tube. Hex nuts against the top and bottom outside surfaces of the tube secured the steel rod tightly in place. The bait was thus detectable by the squirrels, but was not easily removed, reducing the need for frequent maintenance. The placement of the bait midway along the length of the tube ensured that any squirrel attempting to investigate the bait would deposit hairs from both its back and tail onto the overlying glue strip.

At each end of the tube, an additional threaded steel rod extended through a hole in the bottom and approximately 10 cm from the end. We secured each rod with a hex nut against the inside and outside walls of the tube. Each of the 3 rods extended 25 cm below the bottom of the tube. When pressed vertically into the ground, they served as stabilizers, anchoring the tube firmly to the surface of the ground. This hair trap design proved very effective in this application.

At each of the 3 collecting locations (eastern gray squirrels only, Delmarva fox squirrels only, and both eastern gray squirrels and Delmarva fox squirrels), we distributed 20 baited hair traps in a continuous forest stand, with the traps laid out in a 4×5 grid arrangement on a 50-m interval. We left the traps on the ground for 14 days during April 2004, and we checked them occasionally to confirm that at least some of the traps were indeed being visited by squirrels. This time period was arbitrary in the sense that we did not know at the outset how much time would be required for the traps to be discovered and accumulate a useful sample of hairs. On the other hand, we did not want any collected hairs to be exposed to field conditions for an undue period of time. We then retrieved the traps and wrapped the ends with aluminum foil to prevent any exchange of hairs between traps. Wearing a new pair of vinyl gloves for each trap, RDD removed the glue strips from the PVC tubes, placed them in a plastic bag with a unique number, and transported them to N. D. Moncrief (NDM), who was given the uniquely labeled samples without locality information. N. D. Moncrief removed only hairs with follicles from the glue strips with flame-sterilized forceps and severed each hair with flame-sterilized scissors approximately 15 mm from the follicle. Each hair sample consisted of 20 hairs that had follicles.

Voucher specimens, tissues, and hair of Delmarva fox squirrels were transported and are housed at the Virginia Museum of Natural History (VMNH) under Regional Blanket Permit 697823 issued to NDM. Voucher specimens for liver samples were as follows: eastern gray squirrels: Maryland: Allegany County, VMNH 235–244; Dorchester County, VMNH 365, 406–410, 412–413, 1114; Virginia: Alleghany County, VMNH 489–490, 526; Augusta County, VMNH 527–529; Henry County, VMNH 1550–1552, 1554, 1556–1559, 2243. Eastern fox squirrels: Maryland: Allegany County, VMNH 247–252, 254–256; Dorchester County, VMNH 1115–1119, 1121–1124, 1167; Virginia: Alleghany County, VMNH 449–450, 453–454; Sussex County, VMNH 2275–2276.

RESULTS

Of the 10 microsatellite loci surveyed (May et al. 1997), one locus (IGS-110b) produced scoreable products that consistently and unambiguously differentiated eastern gray squirrels and eastern fox squirrels, including Delmarva fox squirrels. Eastern gray squirrels were polymorphic for alleles of size 128–138 base pairs (bp), whereas this same locus was fixed for a single allele of 116 bp in all 56 individual eastern fox squirrels. That is, all 41 Delmarva fox squirrels were homozygous for allele 116. Moreover, the 15 eastern fox squirrels from western Maryland, western Virginia, and southeastern Virginia were also homozygous for allele 116. Therefore, this locus does not distinguish among subspecies of eastern fox squirrels. The 116-bp allele was not present in any of the 56 known eastern gray squirrels we examined.

During the field trial we distributed 20 hair traps at each of 3 locations. In a 2-week period in April 2004, squirrel hairs were deposited on 100% of the traps set at Chincoteague, 80% of the traps set at Patuxent, and 95% of the traps set at Blackwater. Based on the length of individual hairs, we determined that hairs from nontarget species were not deposited on these traps. This was not surprising, given the forested habitat in which we deployed these traps, the diameter (10 cm) of the PVC tubes, and the fact that we secured the glue strips to the inside top surface of the tubes. As much as possible, we selected squirrel tail hairs for DNA extraction because their length made them much easier to handle.

The DNA analysis of hairs collected during the field trials produced the expected results. That is, the sites with Delmarva fox squirrels (Blackwater and Chincoteague) included samples with the 116-bp allele at the IGS-110b locus. This allele was not present in any of the samples from Patuxent, which lacked Delmarva fox squirrels.

Finally, we conducted an experiment to determine whether we could detect Delmarva fox squirrel DNA in mixed samples of hairs from known individuals. Assays we developed were sensitive enough to detect the presence of DNA from a single Delmarva fox squirrel hair when

combined in the same sample tube with DNA obtained from 19 eastern gray squirrel hairs (8 replicates).

DISCUSSION

We have developed a simple genetic test that unambiguously differentiates eastern fox squirrels from sympatric eastern gray squirrels. We have also demonstrated that this test can be applied to hair samples collected noninvasively. The marker we describe herein holds great promise for documenting the presence of Delmarva fox squirrels, even where they are syntopic with eastern gray squirrels.

To address a similar situation, Litvaitis et al. (2006) recently used a noninvasively sampled mitochondrial genetic marker to determine the current distribution of New England cottontails (*Sylvilagus transitionalis*) in a range-wide survey. They amplified species-specific restriction sites in mitochondrial DNA obtained from fecal pellets. This technique allowed Litvaitis et al. (2006) to unequivocally identify localities occupied by New England cottontails, which can be syntopic with eastern cottontails (*Sylvilagus floridanus*). Diagnostic genetic markers have also proven useful for noninvasive detection of other species (Piggott and Taylor 2003, Waits and Paetkau 2005).

Management Implications

This protocol will expedite site review for the Delmarva fox squirrel in connection with the Endangered Species Act consultation process. We suggest that researchers use hair collection devices to survey locations where the presence of Delmarva fox squirrels is suspected but not confirmed by traditional methods such as live-trapping. Researchers can also use this technique in combination with remote sensing to conduct a range-wide survey. We suggest that researchers identify potential habitat by remote sensing (Nelson et al. 2005), then use DNA analysis of samples collected in hair traps to confirm the occupancy of particular locations. These genetic assays are relatively quick and inexpensive (approx. $100/sample), and they can unambiguously identify sites occupied by endangered Delmarva fox squirrels with a minimum investment of funds, time, and effort because handling individuals is unnecessary.

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