

REDISCOVERY OF ROOSEVELT'S BARKING DEER (*MUNTIACUS ROOSEVELTORUM*)

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Data in this study confirm the validity of a species of muntjac, *Muntiacus rooseveltorum*, that has been controversial for 60 years. Diagnostic DNA characters are presented for each species examined including the *M. rooseveltorum* holotype. Three specimens of a recently collected small Laos barking deer have identical sequences to the type specimen of *M. rooseveltorum*. These DNA characters unambiguously diagnose the newly collected specimens as *M. rooseveltorum*. This study highlights the importance of continued field surveys in remote regions and the utility of diagnostic DNA characters in identifying species.

Key words: *Muntiacus rooseveltorum*, Roosevelt's muntjac, diagnostic DNA characters

On 16 May 1929, H. J. Coolidge collected a subadult male muntjac or barking deer in Muong Yo, Laos (ca. 21°30'N, 102°00'E), as part of the Kelley-Roosevelts Asiatic Expedition. Osgood (1932) described this specimen as a new species of muntjac and named it *Muntiacus rooseveltorum*. He remarked on the "curious" combination of cranial characters that made this such an unusual specimen—and completed his species description with "further specimens will be of the highest interest" (Osgood, 1932:192–339).

During the next 60 years, no other specimens were observed, and the validity of this taxon was called into question. Ma et al. (1988) suggested that *M. rooseveltorum* actually might be *M. reevesi*, and in a more detailed analysis, Groves and Grubb (1990) relegated this specimen to a synonym of *M. feae*. Years of warfare and instability had made it difficult to survey and collect wildlife in this region. During the 1990s the resumption of scientific research in the Annamite Mountains, which straddle Laos and Vietnam, yielded a number of startling new

or rediscovered large mammals (Dung et al., 1993; Groves et al., 1997; Schaller and Rabinowitz, 1995; Schaller and Vrba, 1996; Touc et al., 1994). Two sympatric species of muntjac (*M. muntjak* and *Megamuntiacus vuquangensis*) now were known from the forests of the Annamites.

In 1994, two of us (WGR and GBS) were told by local people that there was a third kind of muntjac confined to old-growth forests in this same area, and one of those was observed in January 1995 in a menagerie in the town of Lak Sao, Laos (18°20'N, 105°00'E). That animal was morphologically distinct from the other two described, sympatric species of muntjac (*M. muntjak* and *Megamuntiacus vuquangensis*) but resembled *M. crinifrons* in pelage color (but not in its much smaller size). It also shared some morphological characters with the *M. rooseveltorum* holotype. By January 1996, two nearly-complete skulls and six partial skulls of small muntjac had been obtained in Laos. Genomic DNA was isolated from three of those samples for comparison with the *M. rooseveltorum* holotype and other

TABLE 1.—Sites in 16S rDNA that diagnose *Muntiacus* nucleotide positions 276 and 379 are diagnostic for *M. rooseveltorum* compared with all other *Muntiacus* species. Dots indicate sequence identity to *M. rooseveltorum*. Numbers of individuals sampled for each taxon are indicated in parentheses.

Species	Nucleotide position														
	15	23	46	110	152	157	194	199	240	248	250	254	259	261	263
<i>M. rooseveltorum</i> (n = 4)	T	A	A	A	A	C	A	T	C	A	C	A	T	T	C
<i>M. putaoensis</i> (n = 8)	A	C	.	.
<i>M. truongsongensis</i> (n = 2)
<i>M. vuquangensis</i> (n = 2)	G	T
<i>M. crinifrons/gongshanensis</i> (n = 1)	C	.	.	.	G	T
<i>M. feae</i> (n = 21)	C	.	.	.	G	T
<i>M. reevesi</i> (n = 1)	C	T
<i>M. muntjak</i> (n = 19)	C	T	T
<i>Elaphodus cephalophus</i> (n = 1)	—	G	T	.	T	T	C	A	.
<i>Cervus eldi</i> (n = 1)	C	G	G	G	.	.	G	C	.	A	T

described species of muntjac. Fragments of 16S mitochondrial ribosomal DNA (mt rDNA) were examined for diagnostic sites (Davis and Nixon, 1992; Nixon and Wheeler, 1990) to determine if this taxon represented a new species, a range extension for a previously described species, or the rediscovery of the enigmatic *M. rooseveltorum*. The ability to compare 69-year-old DNA sequences from the only existing specimen with modern samples provided an important opportunity to solve classification of *Muntiacus*.

MATERIALS AND METHODS

The DNA was isolated from 54 muntjac specimens, which provided a wide representation of genetic and geographic variation including species sympatric with the small Laos taxon (Table 1). Most samples consisted of small pieces of dried tissue recovered opportunistically in the field. One sample each of *M. crinifrons* and *M. gongshanensis* was obtained as small bone fragments from museum collections in China. Additional field collected *M. crinifrons* and *M. gongshanensis* samples (n = 14) also were included. A small bit of dried tissue from the *M. rooseveltorum* type specimen was made available from the Field Museum of Natural History, Chicago, Illinois.

The DNA from hair and skin samples was extracted overnight at 56°C in Lifton's buffer (0.1M Tris, 0.2 M sucrose, 0.05 M EDTA, 1%

SDS, pH 8.5) to which 1.4 mg/ml of proteinase K was added. DNA was precipitated using standard phenol-chloroform and ethanol-precipitation procedures (Sambrook et al., 1989). Bone samples were decalcified by soaking in 0.5 M EDTA pH 8.5 for several hours at 37°C prior to extraction and precipitation as above.

Additional procedures that helped to preclude recovery and amplification of any contaminant were used in extracting DNA from the type specimen of *M. rooseveltorum* due to its age, rarity, and frequency of handling by previous researchers. The sample was divided in half to provide two replicate DNA isolations. One replicate was analyzed with the standard protocol described above. The second was analyzed with a modified protocol for vertebrate museum specimens (Rosenbaum et al., 1997). Both extractions were conducted exclusively in the ancient DNA facility of the American Museum of Natural History's molecular laboratory (DeSalle and Bonwich, 1996) to further reduce chances of contamination.

Amplifications were performed in a Perkin Elmer 9600 (cycling conditions: 38 cycles of 94°C denaturation for 45 s, 47°C annealing for 45 s, 72°C extension for 45 s) with the following reagent concentrations: 67 mM Tris, 3 mM MgCl₂, 16.6 mM NH₄(SO₄)₂, 0.8 mM premixed dNTPs, 0.2 uM of each primer and 1 U of *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT). A 551-base fragment of the 16S mt rDNA gene was PCR amplified by use of universal vertebrate primers (Kocher et al., 1989–16Sa

TABLE 1.—*Extended.*

Nucleotide position																					
276	279	283	289	290	322	325	343	351	356	357	361	365	368	373	379	381	393	395	396	406	446
G	T	T	G	T	T	A	T	T	C	C	A	A	T	T	T	T	A	A	—	C	A
A	C	C	.	.	.	—	.	.
A	C	.	A	C	.	.	C	.	T	.	G	.	.	.	C	.	.	.	—	A	.
A	.	.	A	C	.	G	C	—	G
A	.	.	.	C	.	G	C	—	T
A	.	C	.	C	C	C	C	T	.	—	.	.
A	C	C	C	.	.	.	—	.	.
A	C	.	A	C	T	C	C	.	.	.	—	.	.
A	C	.	.	C	C	G	.	.	T	.	G	C	C	T	—	.	.

5'caaacccccgctgtttaccacaaaacat3' [2305] and 16Sb 5'ccggtctgaactcagatcacgt3' [2856]). Numbers in brackets refer to the corresponding positions in *Bos taurus* mtDNA (Anderson et al., 1982; Irwin et al., 1991). PCR products were sequenced in both directions using an ABI automated sequencer. Additional muntjac specific primers were designed for the amplifications of *M. rooseveltorum*. Those included short (ca. 120 base) overlapping internal primers to enable successful amplification from the short strands that usually are isolated from old specimens. Amplification products were visualized by agarose gel electrophoresis and GeneCleaned (Bio101) according to manufacturer's instructions. Purified PCR products were cycle-sequenced using fluorescently labeled dideoxy terminators and run on an Applied Biosystems 373A automated sequencer. Sequences were obtained from both strands and compared analytically. Sequences for multiple individuals of each presumed species were aligned manually. There was only a single gap one base in length in *Megamuntiacus vuquangensis* allowing for constructing an unambiguous alignment by any method. Individual bases, and the haplotype were assessed by Population Aggregation Analysis (Davis and Nixon, 1992) to see if they diagnosed the unfamiliar taxon as a new or previously described species (Nixon and Wheeler, 1990).

RESULTS

Diagnostic DNA characters were identified for each species examined (Table 1) including the *M. rooseveltorum* holotype. Two DNA characters diagnosed *M. rooseveltorum* from all other muntjac. Addition-

al DNA characters differentiated *M. rooseveltorum* from every other species (e.g., 6 sites differ from *M. muntjak*, 11 sites from *Megamuntiacus vuquangensis*). The three specimens of the small Laos barking deer have identical sequences to the type specimen of *M. rooseveltorum*. Those DNA characters unambiguously diagnosed the newly collected specimens as *M. rooseveltorum*. The additional collecting locale (19°49'N, 103°45'E) indicated that *M. rooseveltorum* has a wide, albeit patchy, distribution along the Annamite Mountains in Laos.

DISCUSSION

A great deal of confusion currently surrounds taxonomy of muntjacs (Groves and Grubb, 1990; Ma et al., 1988) reflecting, in part, a lack of morphological differentiation in this group. DNA sequence data from our laboratory suggest that all extant species of muntjac may represent a recent radiation from this ancient lineage of cervids. Eight species generally are recognized (Corbet and Hill, 1992; Schaller and Vrba, 1996). Data in the present study confirm the validity of *M. rooseveltorum*, which has been controversial for >60 years (Groves and Grubb, 1990; Ma et al., 1988).

DNA characters from the 16S mt rDNA proved to be useful for identifying diagnostic characters for the species of muntjac examined. Published studies previously have

demonstrated properties and utility of this gene region (Gatesy et al., 1997; Irwin et al., 1991; Miyamoto et al., 1989). Multiple samples from most taxa allowed us to treat confidently these diagnostic sites as characters, as defined by Davis and Nixon (1992), providing information for species delimitation. In one case, these genetic data question the validity of one taxon (*M. gongshanensis*). *M. gongshanensis* always has been a questionable taxon because it was described based on a single odd karyotype (C. P. Groves, pers. comm.). Here *M. crinifrons* and *M. gongshanensis* are treated as a single taxon because our data from multiple specimens (and other molecular data not presented here) do not diagnose *M. gongshanensis* as distinct.

Yet another muntjac species, *M. truongsoneensis*, recently has been reported from the Annamite Mountains in Vietnam (Giao et al., 1998). Although samples of the newly examined Vietnam animal were not made available for study, DNA sequence data from the cytochrome-b gene (P. Arctander, pers. comm.) and an examination of 16S rDNA from two representatives of this species from Laos (this study) demonstrate that this taxon is diagnosably distinct from all others examined in our laboratory. Although the data presented allow us to confidently announce the rediscovery of *M. rooseveltorum*, additional molecular data are required to assess phylogenetic relationships within the poorly understood genus *Muntiacus*.

The rediscovery of *M. rooseveltorum* as a third sympatric species of barking deer in the Annamite region of Vietnam and Laos suggests that the evolutionary history of this genus is complex. Existence of a fourth species in the Annamite Mountains, *M. truongsoneensis* (one apparently related to *M. rooseveltorum*) highlights the high level of endemism in this region. A better understanding of the highly variable chromosome number found between and within species of muntjac is likely to add additional information about the evolutionary his-

tory of this group (Wurster-Hill and Seidel, 1985). However, such data are difficult or, in the case of a single museum specimen, impossible to gather. Diagnostic molecular characters presented here provide a useful objective framework for such study. With wildlife and habitats disappearing at an alarming rate in Vietnam and Laos, there is an urgent need for additional research.

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LITERATURE CITED

- ANDERSON, S., M. DEBRUIJN, A. COULSON, I. EPERON, F. SANGER, AND I. YOUNG. 1982. Complete sequence of bovine mitochondrial DNA: conserved features of the mammalian mitochondrial genome. *Journal of Molecular Biology*, 156:683-728.
- CORBET, G. B., AND J. E. HILL. 1992. *Mammals of the Indomalayan region*. Oxford University Press, Oxford, United Kingdom.
- DAVIS, J. I., AND K. C. NIXON. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology*, 41:421-435.
- DESALLE, R., AND E. BONWICH. 1996. DNA isolation, manipulation and characterization from old tissues. Pp. 13-22, in *Genetic Engineering* (J. Setlow, ed.). Plenum Publishing Corporation, New York, 18:1-289.
- DUNG, V., P. GIAO, N., N. CHIN, D. TOUC, P. ARCTANDER, AND J. MACKINNON. 1993. A new species of living bovid from Vietnam. *Nature*, 363:443-445.
- GATESY, J., G. AMATO, E. VRBA, G. SCHALLER, AND R. DESALLE. 1997. A cladistic analysis of mitochondrial ribosomal DNA from Bovidae. *Molecular Phylogenetics and Evolution*, 7: 303-319.
- GIAO, P., ET AL. 1998. Description of *Muntiacus truongsoneensis*, a new species of muntjac (Artiodactyla: Muntiacidae) from Central Vietnam, and its conservation significance. *Animal Conservation*, 1: 61-68.
- GROVES, C. P., AND P. GRUBB. 1990. *Muntiacidae*. Pp. 134-168, in *Horns, pronghorns, and antlers* (G. Bubenik and A. Bubenik, eds.). Springer-Verlag, New York.
- GROVES, C. P., G. SCHALLER, G. AMATO, AND K. KHOUNBOLINE. 1997. Rediscovery of the wild pig (*Sus bucculentus*). *Nature*, 386:335.
- IRWIN, D. A., T. D. THOMAS, AND A. C. WILSON. 1991.

- Evolution of the cytochrome b gene of mammals. *Journal of Molecular Evolution*, 32:128–144.
- KOCHER, T. C., ET AL. 1989. Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, 86:377–382.
- MA, S., Y. WANGS, AND C. GROVES. 1988. Taxonomic notes on the subspecies of the Indian muntjac (*Muntiacus muntjak*) in Yunnan, China. *Acta Theriologica Sinica*, 8:95–104.
- MIYAMOTO, M., S. TANHAUSER, AND P. LAIPIS. 1989. Systematic relationships in the artiodactyl tribe Bovini (family Bovidae), as determined from mitochondrial DNA sequences. *Systematic Zoology*, 38:342–349.
- NIXON, K. C., AND Q. D. WHEELER. 1990. An amplification of the phylogenetic species concept. *Cladistics*, 6:212–223.
- OSGOOD, W. 1932. Mammals of the Kelley-Roosevelts and Delacour Asiatic expeditions. Field Museum of Natural History Publications 312, Zoological Series 18:192–339.
- ROSENBAUM, H. C., M. G. EGAN, P. J. CLAPMAN, R. L. BROWNELL, AND R. DESALLE. 1997. An effective method for isolating DNA from historical specimens of baleen. *Molecular Ecology*, 6:677–681.
- SAMBROOK, J., E. F. FRITSH, AND T. MANIATIS. 1989. *Molecular cloning: a laboratory manual*, second ed. Cold Spring Harbor Laboratory Press, New York.
- SCHALLER, G. B., AND A. RABINOWITZ. 1995. The saola or spindlehorn bovid *Pseudoryx nghetinhensis* in Laos. *Orxy*, 29:107–114.
- SCHALLER, G. B., AND E. S. VRBA. 1996. Description of the giant muntjac (*Megamuntiacus vuguangensis*). *Journal of Mammalogy*, 77:675–683.
- TOUC, D., V. DUNG, S. DAWSON, P. ARCTANDER, AND J. MACKINNON. 1994. Science and technology news. Forest Inventory and Planning Institute Hanoi, March 4–13 (in Vietnamese).
- WURSTER-HILL, D. H., AND B. SEIDEL. 1985. The G-banded chromosomes of Roosevelt's muntjac, *Muntiacus rooseveltorum*. *Cytogenetics Cell Genetics*, 39:75–76.

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