GENETIC RELATIONSHIPS DEDUCED FROM CYTOCHROME-*b* SEQUENCES AMONG MOOSE

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ABSTRACT: We studied variation in nucleotide sequences of the mitochondrial cytochrome-*b* gene to assess the phylogeny of moose (*Alces alces*) in general, and the position of North American moose within that phylogeny in particular. We combined North American, Asian, and European haplotypes generated for this study with 3 Eurasian haplotypes obtained from GenBank. No nucleotide variation occurred within moose from North America, whereas 3 haplotypes were present in European moose and 4 haplotypes in Asian moose. Clade structure was consistent over 6 most-parsimonious trees, with Asian haplotypes composing 1 clade, and North American and European haplotypes composing a second, albeit poorly supported clade. Low diversity of nucleotides in cytochrome-*b* indicated a recent ancestry among moose worldwide. Existence of 1 North American haplotype is strong evidence of a single, recent entry into the New World via the Bering land bridge, rather than multiple entries through >1 corridors. Furthermore, no phylogenetic support existed for the theory of distinct lineages of European versus Asian-North American moose.

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Moose (*Alces alces*) arose in Eurasia in the late Pleistocene (Lister 1993), but paleontological (Guthrie 1990) and genetic (Cronin 1992) evidence indicate a recent colonization of North America. Such a recent colonization would result in characteristic genetic signatures in mitochondrial DNA (mtDNA), a haploid genome that is transmitted maternally and is informative for constructing population histories of closely related taxa (Avise et al. 1987). Recently colonized areas would be expected to show less genetic diversity than areas with long-established populations, particularly if the effective size of the founding population was low. Moreover, haplotype composition of a recently founded population would be expected to resemble the composition of the population from which the founders originated. Cronin (1992) analyzed restriction fragment length polymorphisms (RFLP) of mtDNA in North American cervids and reported that moose were unique because they exhibited no detectable variation. There was no comparison to Eurasian moose in that study, however, to determine the degree of difference between moose inhabiting different continents. Therefore, the significance of those findings is difficult to assess.

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Eight subspecies of moose are recognized worldwide: *A. a. alces* in Europe and western Asia, *A. a. pfizenmayeri* in central Asia, *A. a. cameloides* in Mongolia, northwestern China, and southeastern Russia, *A. a. burturlini* in the Russian Far East, *A. a. gigas* in Alaska, USA and the Yukon Territory, Canada, *A. a. andersoni* in western and central Canada and north-central USA, *A. a. shirasi* in the Rocky Mountains straddling the USA-Canada border, and *A. a. americana* in eastern North America (Fig. 1). Flerov (1952) described morphological variation in moose that distinguished western (Europe-western Asia) from eastern (Asia-North America) races. Groves and Grubb (1987) used those differences as justification for labeling eastern and western moose as "semispecies". Eastern and western races coincide generally with differences in karyotype (Boeskorov 1996, 1997), and a length mutation in mtDNA (Mikko and Andersson 1995, Udina et al. 2002). Geist (1998) supported the hypothesis of eastern and western races except to specify that the southern Asian subspecies, *A. a. cameloides*, belonged to a primitive regional fauna and could be considered as a valid subspecies. If differences in morphology and karyotype are based on phylogenetic relationships between races of moose, analysis of mtDNA sequences should detect those differences.

Variation within the nucleotide sequence of the mitochondrial cytochrome-*b* gene (cyt*b*) has been used successfully to examine intraspecific genetic relationships in North American mammals (Talbot and Shields 1996, Demboski et al. 1998). We chose analysis of mtDNA nucleotide sequences rather than RFLP analysis because the former technique has the potential for yielding phylogenies of higher resolution than the latter (Hillis and Moritz 1990). Accordingly, we analyzed nucleotide variation within a section of cyt*b* to examine geographic distribution of genetic variation and to construct a phylogeny for moose.

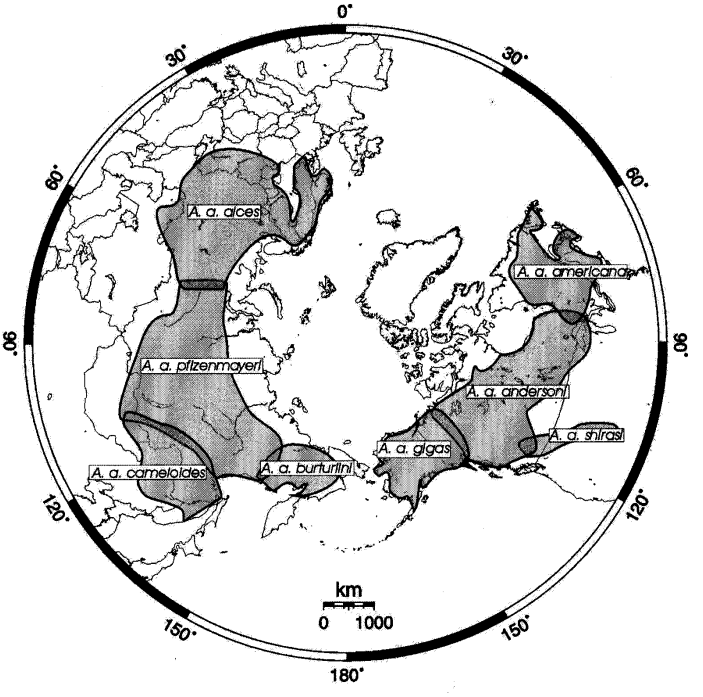


Fig. 1. Polar view of the Northern Hemisphere indicating the approximate distribution of 8 subspecies of moose.

Cyt*b* evolves at a moderate rate in mammals (Irwin et al. 1991, Lopez et al. 1997) and, consequently, often is used in phylogenetic studies of conspecific and congeneric taxa. We used our data to determine if variation within North American moose would be less than variation in Eurasian moose, and if eastern and western races of moose were represented by different lineages of mtDNA.

METHODS

Tissue samples were solicited from moose hunters in Alaska as well as biologists from across North America, Europe, and Asia. Samples were grouped to comprise ≥ 1 population from the range of each North American subspecies, and those populations were combined into continent-level associations. North American subspecies and sampling locations were: *A. a. gigas* (n = 34) from across its range in Alaska; *A. a. andersoni* from central North America (USA: Minnesota, North Dakota, Isle Royale in Michigan; Canada: western Ontario and Manitoba; n = 8); *A. a. shirasi* from Colorado, USA (n = 2); and *A. a. americana* from New Hampshire, USA and New Brunswick, Canada (n = 7). The Colorado population originated from 3 translocations of moose from neighboring states: 12 animals (8 females) from the Uinta Mountains, Utah, USA, in 1978, 12 animals (11 females) from Grand Teton National Park, Wyoming, USA, in 1979, and 12 animals (10 females) from Jackson Hole, Wyoming in 1987 (Duvall and Schoonveld 1988). Asian subspecies and sources were: *A. a. burturlini* (n = 10), which consisted of animals from the Ola Peninsula near Magadan, and the Omolon and Chelomya Rivers, Russia; *A. a. cameloides*, represented by 1 animal housed at a zoo in Harbin, China, and a sequence from GenBank (accession no. AY035872); and *A. a. pfizenmayeri*, consisting of a sequence obtained from GenBank (accession no. AY035873). The European subspecies, *A. a. alces*, consisted of samples collected in Finland (n = 6) and Sweden (n = 6), as well as a sequence of a moose from Norway obtained from GenBank (accession no. AJ000026; Randi et al. 1998).

Tissue samples consisted of skeletal muscle, liver, kidney, skin, blood, or hair. Tissues were stored temporarily at -20°C or preserved in 100% ethanol as soon as possible after collection and were archived at -80°C. All tissue types except blood were subjected to salt extraction for isolation of genomic DNA. DNA from blood was extracted with chelex (Walsh et al. 1991). MtDNA was isolated from nuclear DNA and RNA from 1 moose by means of a $CsCl_2$ density-gradient centrifugation. That sample was used to verify the mitochondrial origin of amplified sequences.

We targeted the 5' region of the cyt*b* gene for analysis. We amplified the sequence with primers MVZ05 (5'—GCAAGCTTGATATGAAAAACCATCGTTG—3') and MVZ04 (5'—GCAGCCCCTCAGAATGATATTTGTCCTC—3') first described by Smith and Patton (1993). Double-stranded templates were amplified in a reaction mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 mM each primer, and 0.5 units DNA polymerase. Cycling conditions were a 2-min soak at 94°C followed by 30 cycles of 94°C (15 sec) denaturation, 50°C (15 sec) annealing, and 72°C (45 sec) extension, followed by one extension period of 10 min at 72°C. PCR products were visualized on a 6% agarose gel with ethidium bromide staining. Cleaned PCR products were cycle sequenced (both directions) with fluorescing ddNTPs. Nucleotide composition of the final products was determined on an automated sequencer (ABI 373, PE Applied Biosystems, Foster City, CA) with standard protocols supplied by the manu-

facturer. Sequences were aligned with the CLUSTAL V algorithm (Higgins et al. 1992) and were edited by visual examination of electropherograms with SEQUENCE NAVIGATOR software (ABI).

We compared nucleotide sequences of individuals and identified sites at which they differed; those data served as the basis for describing individual and population-level variation. Populations also were characterized by haplotype diversity (*H*), which is the probability that 2 randomly selected individuals would have different haplotypes (Nei and Kumar 2000). Nucleotide diversity (π; Nei and Li 1979), which is the probability that 2 homologous nucleotides would differ in 2 randomly chosen individuals, and number of pairwise differences, which is the number of nucleotide substitutions observed between 2 haplotypes, also were determined. Those statistics were estimated with ARLEQUIN software (Schneider et al. 2000). Genetic distances between pairs of populations were computed by applying the Kimura 2-parameter model of sequence evolution (Kimura 1980). Differentiation among all populations was assessed via Φ_{ST}, which incorporates differences in nucleotide and haplotype diversity within and among populations. We analyzed relationships among haplotypes with a maximum parsimony (branch-and-bound search) cladogram, and a neighbor-joining tree (Saitou and Nei 1987) employing 2-parameter distance estimates. Those analyses were performed with the program MEGA version 2.0 (Kumar et al. 2001). Trees were rooted by a haplotype from fallow

Table 1. Nucleotide variation within the first 403 nucleotides of the 5' end of the mitochondrial cytochrome-*b* gene in moose. Only variable nucleotide positions are listed, and dots represent identity with the first sequence. All haplotypes were documented in moose from this study with the exception of Europe3, which was reported by Randi et al. (1998) from Norway. Nucleotide positions were numbered according to the bovine mitochondrial genome (Anderson et al. 1982), with the first nucleotide of cytochrome-*b* numbered 14514. Distributions of haplotypes by sampling location and by subspecies also are indicated.

	Continent			Subspecies								Nucleotide position							
Haplotype	North America	Asia	Europe	*A. a. americana*	*A. a. andersoni*	*A. a. shirasi*	*A. a. gigas*	*A. a. burturlini*	*A. a. cameloides*	*A. a. pfizenmayeri*	*A. a. alces*	14523	14559	14577	14595	14613	14652	14739	14872
North America	55			26	20	2	7					C	T	T	T	T	T	C	C
Asia1		8						7		1		T	C	•	•	•	•	T	•
Asia2		3						1	2			T	C	•	•	•	•	•	•
Asia3		1						1				T	C	•	•	•	C	T	•
Asia4		1						1				T	C	•	•	•	C	•	•
Europe1			4								4	•	•	A	•	•	•	•	T
Europe2			8								8	•	•	A	C	•	•	•	•
Europe3			1								1	•	•	A	C	G	•	•	•

deer (*Dama dama*) obtained from GenBank (accession no. X56290; Irwin et al. 1991), and haplotypes derived for this study from a caribou (*Rangifer tarandus granti*) collected in Alaska (denoted *Rangfer*1) and a reindeer (*R. t. tarandus*) collected in the Omolon River drainage of the Russian Far East (denoted *Rangifer*2). Confidence in the structure of the phylogenies was assessed through 1,000 bootstrap replicates (Felsenstein 1985).

RESULTS

We detected 8 variable sites within the 403 nucleotides of the 5' end of cyt*b*, defining 8 haplotypes (Table 1). Six of the variable sites were transitions and 2 were transversions; the transversions were restricted to European haplotypes. The overall transition:transversion ratio was 7:1 including outgroups and 3:1 for moose only. Six variable sites, including 1 transversion, were synonymous substitutions at the third position of codons, resulting in no substitutions of amino acids in the gene product. In haplotype Europe3, however, 1 transversion occurred at the third position of the thirty-third codon and resulted in the substitution of the amino acid phenylalanine with leucine. The remaining substitution was a synonymous, first-position transition in Europe1. All new haplotypes described in this study, including 2 outgroup haplotypes, were submitted to GenBank and were assigned accession numbers AY090099–AY090107.

We documented an extreme degree of differentiation among continents (Φ_{ST} = 0.89), with no haplotype occurring on > 1 continent (Table 1). We identified 4 Asian, 3 European, and 1 North American haplotypes. Pairwise differences among haplotypes ranged from 1 to 7 substitutions, and associated estimates of genetic distances ranged from 0.2 to 1.8% (Table 2). Estimates of mean (± SD) haplotype diversity for Europe (H = 0.60 ± 0.13) and Asia (H = 0.56 ± 0.11) were similar, as were estimates of nucleotide diversity for haplotypes occurring within those continents (Table 3). The least genetic distance between continents was the comparison between Europe and North America, and the greatest was between Europe and Asia. Europe exhibited the greatest within conti-

Table 2. Genetic distances between pairs of haplotypes for a 403-nucleotide segment of the moose cytochrome-*b* gene. Values above the diagonal are total numbers of substitutions, and those below the diagonal are estimates of substitutions per site using Kimura's (1980) 2-parameter model.

	North America	Asia1	Asia2	Asia3	Asia4	Europe1	Europe2	Europe3
North America		3	2	4	3	2	2	3
Asia1	0.008		1	1	2	5	5	6
Asia2	0.005	0.002		2	1	4	4	5
Asia3	0.010	0.002	0.005		1	6	6	7
Asia4	0.008	0.005	0.002	0.002		5	5	6
Europe1	0.005	0.013	0.010	0.015	0.013		2	3
Europe2	0.005	0.013	0.010	0.015	0.016	0.005		1
Europe3	0.007	0.015	0.013	0.018	0.015	0.007	0.002	

Table 3. Uncorrected distances between continental assemblages of moose haplotypes estimated with cytochrome-*b* (below diagonal). Values in bold along the diagonal are estimates of nucleotide diversity within those assemblages. Kimura's (1980) 2-parameter model of sequence evolution was used in all calculations.

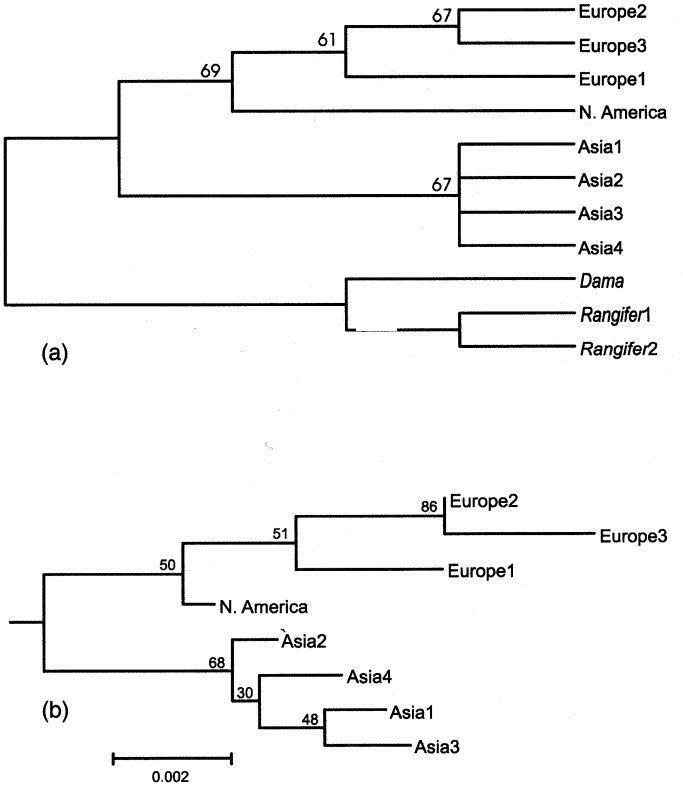
	North America	Asia	Europe
North America	**0.000**		
Asia	0.007	**0.002**	
Europe	0.005	0.012	**0.003**

nent diversity (Table 3).

Haplotype diversity differed among subspecies (Table 1). Although extensive sampling among the 4 North American subspecies did not yield nucleotide diversity, the Eurasian subspecies for which we had >2 samples exhibited great diversity of haplotype composition. *A. a. burturlini* exhibited 4 haplotypes among 10 individuals, and *A. a. alces* exhibited 3 haplotypes among 13 individuals (Table 1). The 2 individuals from *A. a. cameloides* and the single individual from *A. a. pfizenmayeri* exhibited haplotypes that were present in *A. a. burturlini*.

Six most-parsimonious trees were resolved, each with 79 steps (consistency index = 0.91, retention index = 0.90). The

Fig. 2. Phylogenetic trees representing relationships among cytochrome-*b* haplotypes for moose worldwide: (a) strict consensus cladogram from maximum parsimony, showing only those clades that were represented in all most parsimonious trees - numbers listed at nodes represent percent support for that node from 1,000 bootstrap replicates: (b) neighbor-joining phylogram, with bootstrap support indicated at nodes.



strict consensus tree, in which only those clades present in all most-parsimonious trees are displayed, exhibited 2 primary clades of moose haplotypes consisting of a strictly Asian clade and a European-North American clade (Fig. 2a). The neighbor-joining tree exhibited an identical structure (Fig. 2b), although bootstrap support for the clades was weak.

DISCUSSION

Our data are consistent with other studies of genetic variability in cervids that indicate a relative lack of diversity in moose (Wilhelmson et al. 1978, Ryman et al. 1980, Baccus et al. 1983, Cronin 1992), although an instance of high genetic variability in moose has been reported (Hundertmark et al. 1992). Within the same region of cyt*b* that we studied, Kuwayama and Ozawa (2000) reported 32 variable sites among 5 subspecies of North American elk and Euasian red deer (*Cervus elaphus*), and 13 variable sites within 6 subspecies of sika deer (*C. nippon*) restricted to the islands of Japan. The maximum number of substitutions between North American and Asian haplotypes in moose was 4 (all transitions). Comparatively, the minimum difference between haplotypes of North American elk (*C. e. canadensis* = *C. e. nelsoni*) and Asian red deer (*C. e. kansuensis*) was 5 substitutions, 3 of which were transversions (Kuwayama and Ozawa 2000). The magnitude of that difference indicated that, despite similar geographic distributions, North American elk and Asian red deer have been separated longer than North American and Asian populations of moose. The fossil record supports that conclusion (Guthrie 1966, 1990).

Low levels of variability we measured in cyt*b* within continents and small genetic distances between continents indicate a recent common ancestry for moose worldwide. Also, lack of shared haplotypes between continents suggests a small number of founders or bottlenecks. If the number of founding lineages in a continent had been large, we would have expected more haplotypic diversity within continents. Patterns of variation we observed in moose from Asia and Europe were consistent, in each instance, with founding by 1 lineage followed by divergence of 1 or 2 mutations.

Parsimony analysis and genetic distances indicated a closer relationship between North American and European moose than between either of those and Asian moose. Thus, our data provide no support for a fundamental division of moose into European and Asian-North American clades. Rather, the Europe-North America clade was split geographically by the Asia clade, indicating that phylogenetic divergence was not reflected in geographic relationships. That pattern is consistent with a scenario in which moose populations worldwide trace back to recent population expansion combined with small sizes of founding populations (Hundertmark et al. 2002). Absence of variation in cyt*b* in North America is strong evidence for a single colonization characterized by a small effective size. The relatively large haplotype diversity observed in Asian moose likely would have resulted in >1 haplotype in North America if >1 colonization event occurred or if the colonization wave was comprised of many moose.

Our data indicate that Eurasian moose exhibited more diversity than moose from North America, and we find that the spatial distribution of diversity within cyt*b* supports the idea of establishment of continental or regional populations of moose via expansion from small numbers of founders. Moreover, the sharing of 1 haplotype between *A. a. cameloides* and *A. a. burtulini* indicated either recent divergence of those populations or the presence of female-mediated gene flow and provided no evidence of an ex-

treme temporal separation of *A. a. cameloides* from other Asian subspecies. Finally, our data indicate a single entry of moose into North America from Asia, and do not support a fundamental division of moose into European and Asian-North American clades.

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