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ZOOLOGY ====

The Role of the Urals in the Genetic Diversity of the European Moose Subspecies (*Alces alces alces*)

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Abstract—The genetic diversity of the Uralian moose population and the role of the Ural region in the phylogeographic structure of the European moose were evaluated based on sequence polymorphisms of the mtDNA control region. The nucleotide diversity of the Ural moose was low, whereas haplotype diversity was rather high. It was found that the haplotype pool of the Ural moose reflects both the unique features of their mitochondrial lineages and their connection with *Alces alces alces* populations of Europe and West Siberia. The structure of median networks and the territorial haplotype distribution support the hypothesis that the mitochondrial lineages typical for this part of the European moose area originate from a late Pleistocene refugium that was located in the Urals.

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INTRODUCTION

The current level of a species' genetic diversity and the phylogeographic structure patterns result from its evolutionary history and reflect the history of the population dynamics, area growth or fragmentation, species expansion or partial extinction, etc. (Avise et al., 1987; Avise, 2000; Burbrink, 2010). Analysis of polymorphic molecular markers (DNA) can help to elucidate some important events of the species' past and to identify ecological factors that affected most the demographic history of populations and species (Hewitt, 1996, 2000, 2004; Avise et al., 1998; Taberlet et al., 1998). In addition, these data contribute to the understanding of historical ecology.

The moose (Alces alces) is a widespread large Holarctic ungulate species inhabiting Eurasian and North American forests and adjacent ecosystems. In spite of the large number of publications, the systematics of the moose still remains a point of discussion. Based on the differences in morphology and the chromosome number, two forms are conventionally recognized: the European (2n = 68) and the American, or American– Siberian moose (2n = 70); however, it is debated whether these forms should be considered as species (Boeskorov, 2001), races, semi-species, subspecies groups, etc. (Flerov, 1952; Sokolov, 1959; Geptner et al., 1961; Filonov, 1983; Danilkin, 1999; Rozhkov et al., 2009). Most taxonomists hold that Alces is a monotypic genus; this point of view is also supported by genetic data (Hundertmark et al., 2002a, 2002b; Hundertmark and Bowyer, 2004). In contrast to moose inhabiting the Far East, East Siberia, and North America, which are subdivided in several subspecies, moose from Europe, the Urals, and West Siberia (West of the Yenisei) have always been considered as representatives of a single taxon, irrespective of the taxon status assigned. Conventionally, moose from these geographic areas are classified as the European subspecies, *A. a. alces* (Danilkin, 1999).

The genetic diversity and phylogeography of the moose have long attracted researchers' interest, but the amount of information available varies among populations from different parts of the area. Most studies have dealt with the genetic diversity of moose from different parts of North America (Hundertmark et al., 2003; Wilson et al., 2003; Schmidt et al., 2009). The data characterizing East Eurasian moose were obtained from relatively small samples (Udina et al., 2002; Hundertmark et al., 2002a, 2002b). So far, the genetic diversity of A. a. alces has been analyzed in different parts of the moose area: Scandinavia, Poland, the European part of Russia, and West Siberia (Mikko and Andersson, 1995; Udina et al., 2002; Hundertmark et al., 2002a, 2002b; Kholodova et al., 2005, 2008; Charlier et al., 2008; Świsłocka et al., 2008, 2013; Moskvitina et al., 2011; Haanes et al., 2011; Kangas et al., 2013). However, the central part of the subspecies' area, the Urals, has been studied the least. The data on the level and structure of genetic diversity of the Ural moose population are of central importance for comprehending the phylogeographic structure patterns of the European moose. Most studies of moose genetic diversity and phylogeography have been based on sequence polymorphism of the most variable region of the mitochondrial genome: the mtDNA control region (D-loop).

The purpose of the present work was to evaluate the genetic diversity of Ural moose based on sequence polymorphisms of the mtDNA control region and to determine the role of the Urals in the phylogeographic structure of the European moose populations.

MATERIALS AND METHODS

This study was performed with 96 tissue specimens from the Ural moose. These included tooth specimens (n = 71) and wet muscle specimens (n = 25) obtained from moose in Sverdlovsk (n = 49), Perm (n = 45), and Chelyabinsk (n = 2) oblasts in 1996–2012 (Table 1).

DNA was isolated from tooth tissue granules drilled from the midpart of an incisor using a MiniElute PCR Purification Kit (Qiagen, United States) as proposed by Yang et al. (1998) with minor modifications, as well as from muscle tissue using a DNA DiatomPrep 100 kit (Isogen, Russia) as suggested by the manufacturer.

Polymerase chain reaction (PCR) was performed in 20 μ L mixture containing Master Mix X5 reagents and SmarTaq DNA polymerase (Dialat, Russia), as well as LmPro (L15766, 5'-GCCATCAACTC-CCAAAGCT-3') and TDKD (H00074, 5'-CTGA-AGTAGGAACCAGATG-3') primers to a fragment of the moose mtDNA control region (left domain), as described previously (Mikko and Andersson, 1995; Udina et al., 2002).

DNA isolation and preparation of the PCR mixture were performed in safety cabinets preliminarily treated with DNA-EraseTM (MP Biomedicals, United States) under ultraviolet irradiation. During the DNA isolation and amplification experiments, contamination was regularly controlled by using blank control tubes containing ddH₂O instead of DNA. All control reactions were negative.

Nucleotide sequences of the purified PCR products were determined on an automated AB 3130 Gene Analyzer (Applied Biosystems, United States) using a BigDye Terminator kit v. 3.1. (Applied Biosystems) with the forward and reverse primers used in PCR.

The obtained DNA sequences were manually aligned using the Bioedit program (Hall, 1999). Statistical analysis and dendrogram and haplotype network construction were performed with the software packages MEGA 5 (Tamura et al., 2011), Network 4.6.1. (Bandelt et al., 1999), and Arlequin 3.5 (Excoffier and Lischer, 2010). Sequences of the mtDNA control region were compared for moose specimens from different regions of the European part of Russia, including those that were obtained previously (Kholodova et al., 2005; Rozhkov et al., 2009) and in the present work (altogether 108 sequences), as well as the data from the international GenBank database (ncbi).

 Table 1. Distribution control-region mtDNA haplotypes in Ural moose

	Num				
Haplotype	Sverdlovsk oblast (n = 49)	Perm oblast (n = 45)	Chelyabinsk oblast (n = 2)	GenBank acc. no.	
12-SV	16	16	0	KJ960200	
3-SV	12	15	0	KJ960199	
14-SV	11	5	0	KJ960201	
28-SV	3	0	0	KJ960202	
150-SV	3	0	1	KJ960203	
326-P	0	4	0	KJ960204	
11-SV	1	0	0	KJ960205	
VP3-P	0	1	0	KJ960207	
80-SV	1	0	0	KJ960206	
64-SV	1	0	0	KJ960208	
ZL1	0	0	1	KJ960209	
560-P	0	1	0	KJ960210	
408-P	0	1	0	KJ960211	
527-P	0	1	0	KJ960212	
LAC-SV	1	0	0	KJ960213	
LP4-P	0	1	0	KJ960214	

RESULTS AND DISCUSSION

Nucleotide sequences of a 464 bp fragment of the mtDNA control region (D-loop) were obtained for 96 Ural moose specimens. The average nucleotide composition of the fragment studied was as follows: 19.37% cytosine, 33.0% thymine, 36.68% adenine, and 10.96% guanine. Sequence alignment identified 18 substitutions: 16 transitions and 2 transversions. Altogether, 16 haplotypes were described, and their sequences were deposited into the GenBank database (acc. nos. KJ960199–KJ960214). The haplotypes of the Ural moose varied by 1 to 11 bp. Six haplotypes were found in several (2 to 32) specimens, and the others were unique (Table 1). The three most common haplotypes (12-SV, 3-SV, and 14-SV) were present in 78.1% of all Ural moose specimens studied (Table 1).

The phylogenetic relationships among the haplotypes of Ural moose are shown in the median-joined haplotype network (Fig. 1). It contained several closely associated radial structures. Such phylogeographic pattern structures are usually a sign of longterm historical isolation of relatively small groups of animals (Avise, 2000). Due to genetic drift in a small population, a particular haplotype comes to predominate, while mutations accumulating in the course of sufficiently long isolation generate new haplotypes descending from the major one. The haplotype network of the Ural moose featured two pairs of radial structures separated by four mutations, with the central positions occupied by the haplotypes 12-SV-14-SV and 28-SV-3-SV, the most common ones in our population samples. Within each pair of radial structures, central haplotypes were separated by a single mutation. In the neighbor-joining (NJ) dendrogram,



Fig. 1. Median-joined haplotype network for the mtDNA control region (464 bp) of Ural moose. Here and in Fig. 3, circle diameters are proportional to the number of individuals with corresponding haplotypes, and branch lengths reflect the number of mutations between them.

haplotypes comprising the radial structures centered at 12-SV and 14-SV were placed into a separate group with a high level of significance (Fig. 2). The topology of these haplotype networks and the NJ tree reflects the heterogeneity of the haplotype composition of the Ural moose population and suggests that their modern gene pool was formed by confluence of several previously isolated populations of initially low effective sizes, which were due to the small number of founder animals or to the population passing through a prolonged demographic bottleneck.

The genetic distance (*p*-distance) between the haplogroups centered at 12-SV and 14-SV and all other haplotypes of the Ural moose was 1.07%. For the moose, there is still no data concerning the mutation accumulation rate in the mtDNA fragment in question. For this reason, haplotype divergence periods are usually calculated with the data obtained for the European wisent (Burzińska et al., 1999) and cattle (Bradley et al., 1996): 78.5 and 62.8% per Ma, respectively (Hundertmark et al., 2002a; Świsłocka et al., 2013). Calculations based on these coefficients suggest that the Ural moose haplogroups could have diverged approximately 13600 to 17000 years ago. Although these estimates are rather indicative, on the whole they correspond to the last glacial period in the late Pleistocene.

For lack of data, it was not possible previously to establish the role of the Urals in the formation of the phylogeographic structure of the European moose subspecies and to determine to what extent the mitotypic pool of Ural moose is unique or related to *A. a. alces* from other parts of the species' area. To address this issue, we analyzed a combined population sample comprising two similarly sized fairly large samples of moose specimens from the Urals and the European part of Russia. Phylogenetic relationships among the mtDNA haplotypes of these two moose populations are shown in the combined haplotype network (Fig. 3).

On the one hand, the structure of the combined haplotype network showed that the moose populations of the Urals and the European part of Russia are closely related. In particular, the two most common haplotypes that occupied the central positions in the radial structures of the Ural moose haplotype network (12-SV and 3-SV) were also the predominant ones in the European moose population and in the combined sample. Identical haplotypes were previously detected in moose from the European part of Russia (L2 and L1, GenBank acc. nos. KC958905 and KC958904), Finland (Finland4 and Finland3; AF412234 and AF412233), and Northwestern Poland (EU257856, EU257857, EU257848, EU257849) (Hundertmark et al., 2002; Kholodova et al., 2005; Świsłocka et al., 2008). The less common haplotype 28-SV, which also



Fig. 2. Neighbor-joining dendrogram (NJ) constructed for control-region mtDNA haplotypes (464 bp) of the Ural moose using the Tamura–Nei model. Bootstrap support values are given at tree nodes (500 replicates).



Fig. 3. Median-joined haplotype network for the mtDNA control region (464 bp) of the moose population sample including specimens from the Urals (1) and the European part of Russia (2).

BIOLOGY BULLETIN Vol. 41 No. 6 2014



Fig. 4. Frequency distribution of pairwise differences between nucleotide sequences of the mtDNA control region (464 bp) of the Ural moose.

occupies one of the central positions in the median network of Ural haplotypes was also detected in elk specimens from the European part of Russia (L42, KC958910).

On the other hand, certain elements of the mtDNA haplotype pool were specific for the Ural moose. In particular, we described 13 haplotypes that were not present in the European part of the species' area. Among them, haplotype 14-SV was detected in 16 specimens of the population sample studied, 150-SV was detected in three specimens, and the rest were found in individual specimens of Ural moose. Thus, most mtDNA haplotypes of the Ural moose, as opposed to those of the European moose, are unique nucleotide sequences characteristic of this region. It should be noted that one of the Ural haplotypes, 14-SV, was identical to the haplotype L93WS (GenBank acc. no. KJ941196) previously described in a moose from Tomsk oblast in West Siberia (Moskvitina et al., 2011). The fact that the haplotype 14-SV is widespread in the Ural moose population and lies at the center of a radial structure in the median network, as well as that several further Ural haplotypes correspond to the radii of this structure, suggests that it was in the Urals where this haplotype originated and it must have spread to West Siberia in the course of Holocene migration.

On the whole, the repertoire of mtDNA control region haplotypes reflects both the unique nature of mitochondrial lineages of the Ural moose and their connections with *A. a. alces* populations of Europe and West Siberia. Apparently, the phylogeographic structure of moose was shaped by diametrically opposed processes of the two historical periods: the

late Pleistocene was characterized by area fragmentation, decreasing population numbers, and group isolation in refugia, especially during the last glacial maximum, while in the Holocene, during the climate warming and growth of forest ecosystems, the population area expanded, the animal population increased, and isolated groups converged. These processes affected both the mitotype pool structure and the genetic diversity of the Ural moose.

The structure of phylogenetic relationships among haplotypes, as exhibited in the median network topology, as well as the territorial distribution of controlregion mtDNA haplotypes, suggest that in the late Pleistocene the Urals harbored a European moose refugium, wherefrom the unique mitochondrial lineages characteristic of this part of the species' area originate. This notion agrees with the available data on the historical changes in the climate, landscape, ecosystems, and plant and animal diversity of the Urals indicating that, during the last glacial period and the Pleistocene–Holocene transition, the Ural mountains, highlands, and river valleys retained local forest ecosystems that served as refugia for many mammalian species, including the moose (Markova et al., 2008).

As a result of the Holocene confluence of previously isolated moose populations, the modern Ural moose haplotype pool includes several haplogroups and haplotypes common in different parts of the subspecies' area. The bimodality of the pairwise difference frequency distribution also reflects the heterogeneity of the modern mitotype pool of the Ural moose population (Fig. 4).

Region	Ν	VS	ts	tv	nh	H(SE)	π (SE)
Urals	96	18	16	2	16	0.782 (0.025)	0.006 (0.0023)
European part of Russia	108	30	24	6	19	0.716 (0.031)	0.008 (0.0024)
The Urals and the Euro- pean part of Russia	204	39	31	8	31	0.756 (0.021)	0.007 (0.0023)

Table 2. Genetic diversity of moose populations from the Urals and the European part of Russia based on the sequence polymorphism of an mtDNA control region fragment (464 bp)

N, number of specimens; vs, ts, tv, numbers of nucleotide substitutions, transitions, and transversions, respectively; nh, number of haplotypes; *H* and π , haplotypic and nucleotide diversity, respectively; SE, standard error.

According to our data, the gene flow reflecting the directions of animal migrations occurred predominantly from the West to the East. Haplotypes typical for the Urals were not detected in the European part of the area; at the same time, the several haplotypes found in the Ural moose are widespread in the European population, and one haplotype was common with the West Siberian moose but not present in the European part of Russia. Two haplotypes found both in Europe and in the Urals (L1 and L2) were also described in moose from the Yamal peninsula. The predominant direction of gene flow may in part be related to the fact that the European moose population began its expansion earlier than the Ural population. Using Arlequin 3.5 software, we calculated the τ values corresponding to the relative expansion periods for the Urals and the European part of Russia as 5.45 and 6.42, respectively; i.e., in Europe, the moose population began to grow and spread earlier than in the Urals. In addition, in recent centuries, the direction of the population spread could have been affected significantly by the differences in moose population densities due to anthropogenic influence. An analysis of the nuclear microsatellite DNA markers, which reflect both the maternal and the paternal heredity and exhibit high mutation rates, could provide further information on the migration routes of the Ural moose in different periods of their evolution history.

The hypothesis suggesting that the Ural moose population passed through a demographic bottleneck is also supported by the values of the most important characteristics of genetic diversity and their relationship: the nucleotide variation is relatively low, whereas haplotype diversity is fairly high (Table 2). Table 2 also includes the same characteristics for the moose population from the European part of Russia and for the combined population sample. The principal parameters were rather similar among the three samples, which suggests that the genetic diversity and the phylogenetic structure of the moose of both regions were influenced by the same phenomena. It was previously noted that European moose populations of Poland and Scandinavia show a rather low genetic diversity, in comparison to most other common wild ruminant species (Mikko and Andersson, 1995; Hundertmark et

al., 2002a, 2002b; Charlier et al., 2008; Świsłocka et al., 2008). It was also shown that the current genetic diversity of these population exhibits signs of prior population decline and area fragmentation as a result of reducing forest ecosystem areas during the cold period in the late Pleistocene. It has been speculated that the European territory must have contained some Pleistocene refugia.

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