Complex patterns of population genetic structure of moose, *Alces alces*, after recent spatial expansion in Poland revealed by sex-linked markers

Magdalena Świslocka & Magdalena Czajkowska & Norbert Duda & Jan Danyłow & Edyta Owadowska-Cornil & Mirosław Ratkiewicz

Received: 28 September 2012 / Accepted: 21 April 2013 / Published online: 11 May 2013 © The Author(s) 2013. This article is published with open access at Springerlink.com

**Abstract** In recent years, human activity directly and indirectly influenced the demography of moose in Poland. The species was close to extinction, and only a few isolated populations survived after the Second World War; then, unprecedented demographic and spatial expansions had occurred, possibly generating a very complex pattern of population genetic structure at the present-day margins of the species range in Poland. Over 370 moose from seven populations were collected from Poland, and partial sequences of the mitochondrial control region (mtDNA-cr; 607 bp) were obtained. In addition, the entire mtDNA cytochrome *b* gene (1,140 bp) and Y-chromosome markers (1,982 bp in total) were studied in a chosen set of individuals. Twelve mtDNA haplotypes that all belonged to the European moose phylogroup were recorded. They could be divided into two distinct clades: Central Europe and the Ural Mountains. The first clade consists of three distinct groups/branches: Biebrza, Polesie, and Fennoscandia. The Biebrza group has experienced spatial and demographic expansion in the recent past. Average genetic differentiation among moose populations in Poland at mtDNA-cr was great and significant ($\Phi_{ST}=0.407$, $p<0.001$). Using mtDNA-cr data, four separate groups of population were recognized using spatial analysis of molecular variance and principal coordinate analysis, including a relict population in Biebrza National Park, a reintroduced Kampinos National Park population, as well as populations that were descendants of moose that colonized Poland from the east (Lithuania, Belarus, and Ukraine) and the north (former East Prussia). Among all the sequenced Y-chromosome markers, polymorphisms were found in the *DBY14* marker in three populations only; four haplotypes were recorded in total. No significant differentiation was detected for this Y-linked marker among moose populations in Poland. Our mtDNA study revealed that a variety of different factors—bottleneck, the presence of relict, autochthonous populations, translocations, limited female dispersal, and the colonization from the east and north—are responsible for the observed complex pattern of population genetic structure after demographic and spatial expansion of moose in Poland.

**Keywords** Bottleneck · Mitochondrial DNA · Relict populations · Spatial expansion · Translocations · Y-chromosome markers

**Introduction**

Historical processes and geographical factors may strongly affect the genetic structure of mammalian populations (Hundertmark and Bowyer 2004; Pilot et al. 2006). The mechanisms include range shifts, spatial contractions and expansions, the effects of barriers to gene flow, and number and location of refugia (Hewitt 1996, 2000; Excoffier et al. 2009). In recent years, human activity directly and indirectly influenced the demography of free-living animals and, as a result, may have profound consequences for their spatial genetic structure (Nussey et al. 2006; Haanes et al. 2010; Hundertmark and Van Daele 2010). Forest fragmentation and direct persecution by humans have resulted in dramatic
reductions of boreal species’ ranges, and in many cases, present-day populations survived as small isolated groups only (Calvignac et al. 2009). Isolated populations, due to small size and strong genetic drift, exhibit reduced levels of genetic variation and may possess unique mtDNA haplotypes (Rowe et al. 2004).

Human impact on the population genetic structure may also result from introductions and translocations of individuals between populations, especially for rare and endangered species as well as for game animals, like roe deer (Capreolus capreolus) and red deer (Cervus elaphus; Randi 2005; Skog et al. 2009). While the majority of species have suffered from recent environmental changes, there are some mammals that greatly benefited from recent human activity and considerably increased in both number and occupied ranges. Best-known examples are among those that are commensal to humans (Schmölcke and Zachos 2005), but many herbivorous species, especially game animals such as roe deer, red deer, and moose (Alces alces), also expanded their ranges during the last century (Vernes et al. 2002; Charlier et al. 2008; Skog et al. 2009). Factors that allowed such unprecedented range expansions are, for example, climate change, low pressure of predators after the Second World War, and “herbivore-friendly,” present-day management practices in forests (Sommer and Nadachowski 2006; Van Ballenberghe 2006).

Despite the fact that spatial expansions have occurred in the evolutionary history of most species, their impact on population genetic structure has attained surprisingly little attention (Excoffier et al. 2009). The analysis of experimental data as well as simulation study showed that newly colonized areas are expected to be structured into sectors of low genetic diversity separated by sharp allele frequency gradients (Excoffier and Ray 2008). The strong genetic drift usually occurs in populations located on the edge of the expansion; thus, the genetic differentiation among populations should be high. Simulation studies have shown that FST increases sharply during the colonization phase not only if rare long-distance migration events occurred (Nichols and Hewitt 1994), but complex population genetic structure can also emerge through a continuous range expansion without long-distance dispersal (Excoffier and Ray 2008). For example, Coulon et al. (2006), in an empirical study, documented significant genetic structuring of a roe deer population which recently recolonized a fragmented landscape. Genetic structuring was also found among red deer populations in northeastern Poland and was explained by past human management practices and contemporary natural migrations (Niedzialkowska et al. 2012).

Moose exhibit a notable ability to adapt to changing habitats (boreal forest) and underwent large range shifts associated with climate change (Hundertmark et al. 2002). However, as the species is vulnerable to hunting, at the beginning of the nineteenth century, moose populations declined catastrophically from considerable parts of their distribution range in Europe (Schmölcke and Zachos 2005). In countries where current moose populations are abundant, past intense exploitation by humans almost caused the extirpation of the species. In effect, few small and isolated relict populations were left in western Central Europe (Schmölcke and Zachos 2005), including the Biebrza marshes in northeastern Poland (Brincken 1826; Gębczyńska and Raczyński 2004), East Prussia (Steinbach 2009), and Sweden (Charlier et al. 2008). Such relict moose populations most probably experienced bottlenecks; thus, one should expect low intrapopulation diversity and significant genetic differentiation among them. Indeed, studies of the mitochondrial control region (mtDNA-cr) of moose from Sweden and Poland (Hundertmark et al. 2002, Świslocka et al. 2008) confirmed a recent bottleneck in these European populations. These data also supported the relict character of both populations as very distinct mtDNA haplotypes were found in these regions at high frequencies.

The present-day distribution of moose in western Central Europe is a result of spontaneous demographic expansion and successful reintroductions after the Second World War (Raczyński 2006). This expansion was possible due to the formation of pine stands on huge areas of the former Soviet Union and low pressure of predators. Gębczyńska and Raczyński (2004) suggested that numerous immigrants from the east, the increase of an autochthonous Biebrza population (northeastern Poland, <20 individuals after the Second World War), as well as a very successful reintroduction in the Kampinos National Park near Warsaw that started from five individuals (Dźi ciołowski and Pielowski 1993) caused the moose to expand its range in Poland, and some long-distance dispersal events were even documented in Germany (Schönfeld 2009). In addition, numerous moose populations (over 1,500 individuals) survived the Second World War and stayed in 1945 in East Prussia (Steinbach 2009). This population declined catastrophically soon after the Second World War due to poaching; however, some individuals could have survived near the Russian/Polish border and contributed to the present-day gene pool of the species.

If all the aforementioned populations were involved in the spatial expansion of moose, one should expect very complex patterns of population genetic structure with sharp allele frequency gradients at current margins of the species range in Poland.

In this study, we analyzed mitochondrial DNA sequences (control region and cytochrome b gene) as well as nine Y-chromosome markers (Y-chromosome-conserved anchor-tagged sequences (YCATS) and SRY gene) in moose originating from seven populations in Poland, where today the species reaches its natural westernmost edge/range. These sex-linked DNA markers were confirmed to be useful in genetic approaches of mammalian populations (Hellborg and Ellegren 2004).
Our two main objectives were (1) to investigate in more detail phylogenetic relationships among mtDNA haplotypes of moose within the European haplogroup and (2) to reveal the pattern of genetic differentiation among moose populations in Poland after a spontaneous and human-mediated range expansion after the Second World War. We predicted that this expansion from different sources should lead to both high haplotype diversity and high levels of population differentiation. Special emphasis was also given to the autochthonous, possibly relict group of moose from the Biebrza National Park, and we estimated parameters for spatial and demographic expansion of the mtDNA haplotype group that possibly originated in this area.

Material and methods

We analyzed DNA samples of 377 moose collected over the years 2007–2011 from seven localities in Poland (Fig. 1): the Biebrza National Park (53°24′25″ N, 22°47′43″ E), the Krzyzyn Forest (53°10′35″ N, 23°48′47″ E), the Augustow Forest (53°58′46″ N, 23°17′23″ E), the Srokowo State Forest (54°12′49″ N, 21°31′15″ E), the Gostynin-Wlochawek Forests (52°38′54″ N, 19°04′04″ E), the Kampinos National Park (52°18′00″ N, 20°49′48″ E), and the Polesie National Park (51°23′37″ N, 23°11′41″ E; Table 2). Samples were collected within the moose core area in Poland inhabited by about 70% of the population. Samples were composed of muscles and skin from individuals killed in car accidents or poached. Stool samples were also collected in winter (individuals were identified using 11 microsatellite loci; data not shown). Samples represented a mixture of males, females, and individuals of unknown sex. They were stored at −20 °C, after which the tissue samples were extracted with the DNeasy Blood and Tissue Kit; for stool samples, the QIAamp DNA Stool Mini Kit (Qiagen, Germany) was used.

Two classes of DNA markers were used in further analyses: mitochondrial DNA and Y-chromosome markers. Amplification of 607 bp of the left hypervariable domain of the mitochondrial control region and a portion of the tRNApro gene was performed using primers LGL283 and ISM015 (Hundertmark et al. 2002; Table 1) and used in phylogenetic and population genetic analyses. The entire mtDNA cytochrome b (1,140 bp long) gene was PCR-amplified in a chosen set of individuals for phylogenetic analyses using primers ML103 and MH104 (Chikuni et al. 1995; Table 1). We used eight universal markers, YCATS, that were previously amplified in males of roe deer and reindeer (Rangifer tarandus; Hellborg and Ellegren 2003). We obtained PCR products for DBY9, DBY14, DBY17, DBY18, DBY19, DBY14, and UTY11, but not for UBE1Y6 and UTY5 markers (Table 1). In order to verify possible polymorphisms found using universal primers, we designed moose-specific primers with the FastPCR software (Kalendar et al. 2009) to amplify the DBY9 and DBY14 markers (Table 1). Moose-specific primers were also designed to amplify 472 bp of a fragment of the SRY gene for genetic sex identification. These seven Y-chromosome markers were sequenced in 110 males from six populations studied (N ranged from 8 to 37 bulls).

PCR were performed with a GeneAmp PCR System 9700 (Applied Biosystems) in 5-μL reaction volumes containing 2 μL genomic DNA (−20 ng), 1.7 μL Qiagen Multiplex PCR Master Mix (1×), 0.3 μL mix of primers (0.2 μM of each primer), and 1 μL RNase-free water. The reaction conditions were as follows: 15 min at 95 °C of an initial denaturation, 35 cycles with denaturation at 94 °C for 30 s, annealing at 57 °C (profile A) or 50 °C (profile B; see Table 1) for 90 s, extension at 72 °C for 60 s, and final elongation for 30 min at 60 °C. Sequencing reactions in both directions were performed using the BigDye™ Terminating Cycle Sequencing Kit (Applied Biosystems) following the manufacturer’s protocol. The detection of sequencing reaction products was carried out on an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were aligned manually in the BioEdit sequence-editing program (Hall 1999). The sequences of all haplotypes were submitted to GenBank and assigned the following accession numbers: KC337263–KC337269 (for mtDNA-cr), KC337270–KC337273 (for the mtDNA cytochrome b gene), and KC337279–KC337282 (for the DBY14 marker).

Phylogenetic analyses

To test the phylogenetic relationships among the concatenated control region and cytochrome b haplotypes, we constructed phylogenetic trees with Mega v.5.05 (Tamura et al. 2011) with 1,000 bootstrap replicates used to assess support for tree nodes. The optimal model of substitution (Hasegawa et al. 1985) for our mtDNA sequences was determined using Akaike’s information criterion (Akaike 1973) with jModelTest (Posada 2008) and used to calculate a maximum-likelihood (ML) algorithm. A neighbor-joining (NJ) tree of haplotypes was computed with Kimura’s (1980) model of sequence evolution, which is the closest available MEGA model to HKY. Trees were rooted using mitochondrial sequences from reindeer (AB245426) and water deer (Hydropotes inermis; NC011821), downloaded from GenBank. We also created a descriptive median-joining network with default settings for control region mtDNA haplotypes using the program Network v.4.6.1.0 (Bandelt et al. 1999). To construct a median-joining network, apart from the mtDNA-cr haplotypes found in our study, we used moose haplotypes previously published by Hundertmark et
**Fig. 1** Study area, sampling sites, and frequency of 12 control region mtDNA haplotypes found in the moose populations studied. *Gray background on the map shows moose distribution in Poland.* *GWF* Gostynin-Wlochawek Forests. *Relict moose population in Biebrza NP.* **Translocated moose population in Kampinos NP.**

| Table 1 | PCR primers and PCR profiles used for the amplification of particular genes |
| --- | --- |
| Gene | Primer | PCR primer sequence 5′–3′ | N | Length (bp) | Profile of PCR reaction | Source |
| --- | --- | --- | --- | --- | --- | --- |
| mtDNA-cr | LGL283 | TACACTGGTCTTGTAAAC | 377 | 607 | Profile A | Hundertmark et al. (2002) |
| | ISM015 | ATGGCCCTGTAGAAAGAAC |  |  |  |  |
| cytb mtDNA | ML103 | GACTAATGATATGAAAAACATCGTTG | 11 | 1,140 | Profile A | Chikuni et al. (1995) |
| | MH104 | TTGTCTTTCTCTCTTTTACAGAC |  |  |  |  |
| DBY4 | DBY4-F | TGATGTTATTGGYRRCGTGA | 15 | 214 | Profile B | Hellborg and Ellegren (2003) |
| | DBY4-R | CCGTTGCTCTACTGTTATA |  |  |  |  |
| DBY7 | DBY7-F | GTTCAGGAGARGCTTTTGAA | 30 | 265 | Profile A | Hellborg and Ellegren (2003) |
| | DBY7-R | CAGCCAATTCTTTGTTG |  |  |  |  |
| DBY8 | DBY8-F | CCCCCAACAGAGAATGGCT | 25 | 138 | Profile A | Hellborg and Ellegren (2003) |
| | DBY8-R | CAGCCCACTAATACTACA |  |  |  |  |
| DBY9 | DBY9-F | CTAGAGTTGCTCTTGTGGA | 17 | 418 | Profile A | Hellborg and Ellegren (2003) |
| | DBY9-R | AATCCCTATTCCAGC |  |  |  |  |
| | DBY9_alcesF | ATTAGACGTGAGTGACTTGTA | 14 | 194 | Profile A | This study |
| | DBY9_alcesR | CATACAGATCTAATAACAATTAGCT |  |  |  |  |
| DBY14 | DBY14-F | CAAGAACTGCCTCTTTGGTG | 29 | 296 | Profile A | Hellborg and Ellegren (2003) |
| | DBY14-R | GCTCCAAATTCCTCCACTG |  |  |  |  |
| | DBY14_alcesF | CCATACCTGTAAGTGAC | 110 | 154 | Profile B | This study |
| | DBY14_alcesR | AAATCTCCACTGAA |  |  |  |  |
| UBE1Y6 | UBE1Y6-F | CCCCTGCACACCKRCAT | 8 | NP | Profile A | Hellborg and Ellegren (2003) |
| | UBE1Y6-R | AAGCCGAATTGATRAARTC |  |  |  |  |
| UTY5 | UTY5-F | TTGTTTTTCTCTAYTTTCATA | 8 | NP | Profile A | Hellborg and Ellegren (2003) |
| | UTY5-R | GTGTCAACATAAAGGACRTCT |  |  |  |  |
| UTY11 | UTY11-F | CATACAATTTTGATAAATCCCAA | 18 | 545 | Profile B | Hellborg and Ellegren (2003) |
| | UTY11-R | TGGTAGAAAGAATGTCGAGA |  |  |  |  |
| SRY | SRY-alcesF | TGTTGACAGTAGTGAACGATGTT | 16 | 472 | Profile A | This study |
| | SRY-alcesR | TATTGAGATAAACGGCAGAAGTACGACT |  |  |  |  |

*NP* no product

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al. (2002), with the following GenBank accession numbers: AF412253 (H23), AF412254 (H24), and AF412261 (H25).

We calculated net pairwise divergence ($d_\delta$) between mtDNA clades—Central Europe and Ural—and among branches within the clade Central Europe using the program Mega v.5.05. The standard error of estimates was calculated with 1,000 bootstrap pseudo-replicates. We used a Bayesian approach implemented in BEAST v1.7.2 software (Drummond et al. 2012) to calculate the time of divergence between European and Asian moose, and also between two distinct European clades (Central Europe and Ural), and between particular groups/branches from clade Central Europe for concatenated control region and cytochrome $b$ sequences. A strict molecular clock was applied with previously reported mutation rates for control region (4 and 8 % Ma) and cytochrome $b$ (2 % Ma) in ungulates (Randi et al. 1998).

Due to the fact that the mutation rate has not been calibrated for moose, we also used a mutation rate of 31.4 % per million years for mtDNA-cr derived for domestic cattle (Bradley et al. 2002). The significance of $\Phi$ statistics was tested using 10,000 permutations for $K=2$ to $K=6$ partitions of the sampling sites. Principal coordinate analysis (PCA) was performed on mtDNA $\Phi_{ST}$ data in GENALEX v.6.0 (Peakall and Smouse 2006).

Results

The analysis of a 607-bp mitochondrial DNA control region fragment amplified from 377 moose in Poland (Fig. 1) yielded 12 haplotypes (GenBank accession numbers for new haplotypes: KC337263–KC337269) as defined by 27 polymorphic sites (all being transitions). Five haplotypes were found in recent studies, while the other seven were previously unreported. We detected the H1 haplotype (GenBank accession no. EU257814) that was described for the first time from the Biebrza NP (Swislocka et al. 2008) and the H2–H4 haplotypes reported by Hundertmark et al. (2002) (Finland 1–3; GenBank accession nos. AF412231–AF412233). Interestingly, we also found the haplotype H6 (GenBank accession no. AF412230) that was found by the latter authors in moose from Sweden. The sequence analysis of a whole cytochrome $b$ gene (1,140 bp) in a selected group of moose that had different mtDNA-cr haplotypes revealed in total five synonymous substitutions (all being transitions) that defined four different haplotypes. The phylogenetic analysis (Fig. 2) of the concatenated control region and cytochrome $b$ haplotypes revealed that all mtDNA haplotypes detected in Poland belong to the European haplogroup of moose (Hundertmark et al. 2002). A network (Fig. 3) was also drawn using the median-joining method to identify possible relationships between 12 mtDNA-cr haplotypes found in this study and other control region mtDNA sequences downloaded from GenBank. Two haplotype clades within the European haplogroup of moose were distinguished in Poland: Central Europe and Ural (Figs. 2 and 3). Notably, these clades had high bootstrap support on phylogenetic trees (Fig. 2). In addition, the clade Central Europe consists of three distinct groups/branches: Biebrza (haplotypes H1, H10, and H13); Polesie (haplotypes H12 and H20 in West Polesie Biosphere Reserve); and Fennoscandia (Scandinavian moose—haplotypes H6 and H11 in Kampinos and haplotypes H17 and H22 in former East Prussia). The Fennoscandia group might be further divided into Scandinavian and East Prussia branches (Fig. 2). The net divergence ($d_\delta$) between clades Central Europe and Ural was 0.7 %, while that among three distinct groups from the clade Central Europe ranged from 0.8 % (Polesie vs. Fennoscandia) to 1.3 % (Biebrza vs. Fennoscandia). Our estimates of divergence based on
mutation rates of 4 and 8% for the control region and 2% for cytochrome b between European and Asian moose were 0.33 Ma (95% highest posterior density interval (HPD) = 0.20–0.48 Ma), between clades Central Europe and Ural of 0.14 Ma (95% HPD = 0.08–0.21 Ma), and between groups Biebrza and Fennoscandia of 0.13 (95% HPD = 0.07–0.20 Ma). When we applied the mutation rate of 31.4% per million years for mtDNA-cr derived for domestic cattle, the divergence values between European and Asian moose were 67,000 years ago (95% HPD = 40,800–97,000), between clades Central Europe and Ural of 47,000 years ago (95% HPD = 28,000–69,500), and between groups Biebrza and Fennoscandia of 46,800 years ago (95% HPD = 26,000–69,500).

Demographic expansion was tested using mtDNA-cr sequences and detected in the Biebrza group of moose. Tajima’s neutrality test was negative and significant ($D = -1.975, p < 0.001$), while Fu’s value was also negative, albeit non-significant ($F_S = -1.312, p = 0.266$). The mismatch distribution fits unimodal curves expected under the sudden expansion model (rangedness index, $r = 0.393, p = 0.700$). The demographic expansion event occurred at $\tau = 3.0$ and involved a population change from an initial $\theta_0 = 0$ (95% CI = 0–0.007) to a final $\theta_1 = 0.272$ (95% CI = 0–99,999), albeit the 95% CI overlapped. Assuming a mtDNA-cr mutation rate of 0.04–0.08 substitution per site per million years and a generation time of 7 years, the demographic expansion of the Biebrza group took place 4400–8800 BP (95% CI = 490–10,300). The spatial expansion of the Biebrza moose in Poland occurred very recently ($\tau = 0.109$), corresponding to 160–320 years ago (95% CI = 91–4,600).

In the moose populations studied, the number of mtDNA-cr haplotypes ranged from three to six (Fig. 1 and Table 2). The highest genetic diversity was found in Srokowo and Gostynin-Włocławek ($h = 0.757$ and 0.776; $\pi = 1.41$ and 1.30, respectively), whereas the corresponding values were the lowest in the Biebrza and Augustów populations. Despite the fact that the number of haplotypes per population did not exceed six, the number of segregating sites within a given population ranged from 14 to 22, indicating that the majority of haplotypes were not phylogenetically close.

Based on an assessment of the mtDNA-cr haplotype frequencies within the Biebrza population, we distinguished three genetically distinct subpopulations that correspond to three Biebrza valley basins: (1) upper (UBB), (2) middle (MBB), and (3) lower (LBB). All the Biebrza subpopulations differed significantly with respect to haplotype frequencies (at $p < 0.05$). The corresponding $F_{ST}$ values were: 0.23 (UBB vs. MBB), 0.59 (UBB vs. LBB), and 0.09 (MBB vs. LBB). In the upper Biebrza basin, $H_1$ was the most
frequent haplotype. In effect, the UBB subpopulation of moose resembled more the Augustów population than other Biebrza subpopulations (MBB and LBB).

Pairwise genetic differentiation values (mtDNA-cr) between moose populations ranged from 0 to 0.552 ($F_{ST}$) and from 0 to 0.611 ($\Phi_{ST}$); the majority of comparison values (95 %) were significant (Table 3). The only $F_{ST}$ value that did not differ significantly from zero was between Knyszyn and Polesie populations, indicating their common origin. Non-significant values of $\Phi_{ST}$ were also found between Knyszyn and Polesie as well as Srokowo and Gostynin-Wloclawek Forests (not studied for Y-linked markers).

### Table 2 Molecular diversity indices for the moose populations in Poland

| Number | Population      | N     | Percentage | $N_h$     | $\pi$ (±SE) | $\sigma$ | $\Phi$ | $M$         |
|--------|-----------------|-------|------------|-----------|-------------|---------|--------|-------------|
| 1.     | Biebrza NP      | 155   | 16         | 4         | 0.330 (±0.04) | 0.65 (±0.36) | 16     | 3.97 (±2.00) | 0.53 (0.001–2.30) |
| 2.     | Knyszyn F       | 18    | 14         | 3         | 0.627 (±0.06) | 0.79 (±0.45) | 14     | 4.81 (±2.46) | 1.73 (0.13–7.13) |
| 3.     | Augustów F      | 34    | 10         | 4         | 0.485 (±0.09) | 0.59 (±0.34) | 16     | 3.57 (±1.88) | 0.96 (0.001–3.77) |
| 4.     | Srokowo F       | 17    | 26         | 6         | 0.757 (±0.09) | 1.41 (±0.77) | 22     | 8.57 (±4.17) | 3.15 (0.74–10.63) |
| 5.     | GWF              | 37    | 23         | 6         | 0.776 (±0.03) | 1.30 (±0.68) | 20     | 7.86 (±3.84) | 3.18 (0.80–9.97) |
| 6.     | Kampinos NP     | 67    | 56         | 6         | 0.473 (±0.07) | 0.69 (±0.38) | 21     | 4.18 (±2.10) | 0.94 (0.03–3.39) |
| 7.     | Polesie NP      | 49    | 33         | 4         | 0.652 (±0.04) | 0.88 (±0.48) | 15     | 5.36 (±2.63) | 1.90 (0.25–5.44) |
|        |                 | 15    | 1          | 1         | 0.000 (±0.00) | 0.00 (±0.00) | 0      | 0.00 (±0.00) | –             |
|        | All             | 377   | 12         | 12        | 0.750 (±0.01) | 1.20 (±0.62) | 27     | 7.26 (±3.41) | 1.77 (0.28–6.09) |
|        |                 | 110   | 4          | 4         | 0.106 (±0.04) | 0.11 (±0.16) | 4      | 0.16 (±0.22) | –             |

$N$ sample size ($N$ for mtDNA-cr data is always larger than for males analyzed for the $DBY14$ marker), $N_h$ number of haplotypes, $h$ haplotype diversity, $\pi$ nucleotide diversity (in percent), $S$ number of segregating sites, $PD$ mean number of pairwise differences, $M$ migration parameter calculated from spatial expansion (95% CI), SE standard error, $GWF$ Gostynin-Wloclawek Forests (not studied for Y-linked markers).

*Percentage of estimated census size*

Out of nine Y-linked markers studied (1,982 bp in total), two ($DBY9$ and $DBY14$) were found to be variable in male moose from Poland using universal primers (Hellborg and Ellegren 2003). However, when we used moose-specific primers for both markers, $DBY9$ turned out to be monomorphic and $DBY14$ was the only one variable. For this marker, we found four haplotypes among the 110 studied bulls (GenBank accession nos. KC337279–KC337282) as defined by four polymorphic sites (two transitions and two
transversions). The DBY14-H1 haplotype differed by one substitution from the DBY14-H2 haplotype; DBY14-H2 differed by one substitution from DBY14-H4. Two substitutions distinguished DBY14-H1 from DBY14-H3 and DBY14-H1 from DBY14-H4. The DBY14-H1 haplotype was the most common and was fixed in Augustów, Polesie, and Srokowo populations. Two DBY14 haplotypes were present in Biebrza (f(DBY14-H1)=0.95, f(DBY14-H2)=0.05) and Knyszyn (f(DBY14-H1)=0.91 and f(DBY14-H3)=0.09). Interestingly, as many as four DBY14 haplotypes (H1–H4) were found in the reintroduced Kampinos population: f(DBY14-H1)=0.88, f(H2, H3, H4)=0.04 (each). Haplotype (h) and nucleotide (π) diversity in the overall male moose sample in Poland for the DBY14 marker were 0.106 and 0.11 %, respectively. All pairwise genetic differentiation values (both FST and ΦST) between moose populations for the DBY14 marker ranged from 0 to 0.05 and were not significantly different from zero.

**Discussion**

This study using sex-linked markers (mtDNA and YCATS) demonstrated considerable genetic variation of moose in Poland. As many as 12 mtDNA-cr haplotypes were found in a total sample, which corresponds to about 71 % of control region haplotypes recorded in the European phylogroup of the species so far (Hundertmark et al. 2002; M. Niedzialkowska, personal communication). Notably, seven haplotypes were described for the first time, indicating Poland as an important area of moose haplotype diversity. The relatively high mtDNA-cr diversity of moose in Poland may be due to several non-mutually exclusive factors: (1) the large number of founding individuals colonizing Poland from the east (Lithuania, Belarus, Ukraine) after the Second World War; (2) the presence of small local populations at few sites and admixture after spatial expansion; and (3) successful reintroduction(s). Indeed, pine stands planted on huge areas of the former Soviet Union that started soon after the Second World War allowed an unprecedented increase in moose which resulted in spatial expansion to the west (Poland, Czech Republic, and Germany; Gębczyńska and Raczyński 2004; Schönfeld 2009). The colonization from the former Soviet Union is clearly visible by the presence of the H2, H3, and H4 mtDNA-cr haplotypes at relatively high frequencies, especially in populations situated close to the eastern border of Poland (Knyszyn, Augustów, Forests and Polesie; Fig. 1). In addition, large numbers of moose (about 1,500) were present in 1945 in East Prussia (present-day Kaliningrad Region, Russia; Steinbach 2009), and some of them could have colonized Poland by crossing the Russian–Polish border. This was probably evidenced by Lenkowa and Panfil (1973) who reported that, soon after the Second World War, a few individuals were present near Gól (northern Poland). Thus, individuals possessing the haplotypes H17 or H22 that were found in the Srokowo State Forest could have partly originated from the East Prussia moose population. Interestingly, haplotypes of these moose formed a branch within Fennoscandian moose that grouped with Scandinavian mtDNA haplotypes. Moose also survived the Second World War in Poland, but their number was very low (<20 individuals at the Biebrza marshes, NE Poland; Tomek 1977; Dzieniołowski and Pielowski 1993). According to Gębczyńska and Raczyński (2004), this population could be a relict group of its previous Holocene range that split early

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**Table 3** Genetic differentiation for mtDNA-cr between moose population pairs as measured by ΦST (below diagonal) and FST (above diagonal)

| Population       | Biebrza NP | Knyszyn F | Augustów F | Srokowo SF | GWF | Kampinos NP | Polesie NP |
|------------------|------------|-----------|------------|------------|-----|-------------|------------|
| Biebrza NP       | –          | 0.519     | 0.611      | 0.389      | 0.234 | 0.547       | 0.436      |
| Knyszyn F        | 0.507      | –         | 0.092      | 0.119      | 0.139 | 0.459       | 0.000      |
| Augustów F       | 0.552      | 0.121     | –          | 0.181      | 0.267 | 0.532       | 0.176      |
| Srokowo SF       | 0.452      | 0.110     | 0.071      | –          | 0.042 | 0.292       | 0.130      |
| GWF              | 0.467      | 0.131     | 0.180      | 0.101      | –    | 0.249       | 0.111      |
| Kampinos NP      | 0.588      | 0.434     | 0.475      | 0.388      | 0.259 | –           | 0.415      |
| Polesie NP       | 0.433      | 0.000     | 0.187      | 0.152      | 0.154 | 0.409       | –          |

Statistical significance, p<0.01. Non-significant values are given in italics

GWF Gostynin-Włocławek Forests

![Fig. 4 Principal coordinate analysis performed on pairwise ΦST values of the studied moose populations in Poland](image-url)
from the main wave of colonization. This was partly confirmed by Świslocka et al. (2008) who found a unique haplotype (H1), distinct from others, among animals from this area at high frequency. In this study, we not only confirmed the presence of the H1 haplotype at very high frequency in the Biebrza population but also found two other haplotypes (H10 and H13) that clustered together with the H1 haplotype on the neighbor-joining tree with high bootstrap support (Fig. 2). These haplotypes, however, were not present in the Biebrza population but also found two other haplotypes (H10 and H13) that clustered together with the H1 haplotype in Poland before a severe bottleneck occurred at the end of the nineteenth century (Brincken 1826). Genetic uniqueness of the Biebrza population is somewhat strengthened by the fact that we found the DBY14-H2 allele in 5% of males; this allele was not recorded in the other studied populations, except for the Kampinos population where four DBY14 haplotypes were found. Generally, low levels of Y-chromosome polymorphism have been recorded in different species of natural mammalian populations (Hellborg and Ellegren 2004). The authors found reduced levels of genetic variability on the Y-chromosome among all studied species, but one of the lowest levels were detected in reindeer and cattle species, as well as lynx (Lynx lynx), where the surveyed Y-chromosome sequences were completely monomorphic. Lack of variation was also found in Y-chromosomal introns of red deer (Barbosa and Carranza 2010). Thus, Y-chromosome polymorphism in moose with four haplotypes that were not randomly distributed in Poland is rather unexpected when compared to other ungulate species.

The Kampinos population arose from a successful introduction of five individuals (two bulls and three females) from Belarus in 1951 (Dzięciołowski and Pielowski 1993). The successful management efforts allowed this population to increase in number and to expand. It is very probable that due to the founder effect, the mtDNA-cr H11 haplotype increased in frequency considerably despite the fact that in Belarus it is very rare (M. Niedzialkowska, personal communication). The presence of six mtDNA-cr and four DBY14 haplotypes in the Kampinos population cannot be explained by the introduction of five individuals only. Indeed, there is also evidence of the translocation of at least a single bull from the Białowieża enclosure in 1955 that was a descendant of Swedish moose, or less likely from Siberia when the Białowieża reintroduction (based on Swedish, Belarusian, and, possibly, Siberian moose; Karpiński 1951) failed. The signs of this theoretically unsuccessful reintroduction are, however, still present as the H6 haplotype, typical for moose in Sweden, was found in the Kampinos population, albeit at a very low frequency (in a single moose). Moreover, long-distance dispersal of both female and male moose was evidenced from the Biebrza to Kampinos population as the mtDNA-cr H1 and DBY14-H2 haplotypes were recorded in this reintroduced population. Thus, our study demonstrated that even reintroduced populations that originated from very limited numbers of founders, due to admixture of individuals from local populations and/or long-distance dispersal, could harbor unprecedentedly high levels of genetic polymorphism. The origin of Polish moose from various sources is supported by the relatively high number of haplotypes and possible presence of two separate mtDNA clades (termed Central Europe and Ural) that all belonged to the European phyllogroup (Fig. 2). We also found three distinct branches within the clade Central Europe in our country (Biebrza, Polesie, and Fennoscandia). It is worth stressing that the same tree topology was obtained using the Bayesian method. The topology of haplotype network by possessing long terminal branches and comb-like structure clearly shows signs of their recent expansion. Excoffier and Ray (2008) suggested that recently colonized areas due to spatial bottlenecks might exhibit private phylogenetic lineages. This does not rule out the possibility that some moose have survived in several distinct refuge areas in Europe as paleontological data evidenced that moose was present in the Balkans, Carpathian, and Italian Peninsula refugia during the Last Glacial Maximum (Sommer and Nadachowski 2006). Our estimate of the divergence time for European and Asian moose ranged from 201,800 to 480,400, suggesting that these two haplogroup divergences predated the last glaciation. This is a general phenomenon for species in Europe revealed by mtDNA (Taberlet et al. 1998). The defined clades of moose (Central Europe and Ural) from the European lineage could have evolved during the Riss Glacial Period, as suggested by estimates of divergence times (140,000 years ago). Three distinct branches within the clade Central Europe could have evolved further during the glaciations. Our results are consistent with the divergence times for the different haplogroups detected in red deer (Skog et al. 2009); however, they considerably predate the estimate reported by Hundertmark et al. (2002). The authors reported $d_{A}$ between the European and Asian phyllogroups (2.5%) of moose and postulated that they diverged approximately 34,000 years ago. However, they assumed a mutation rate ($\mu$) of 31.4% per million years derived for domestic cattle (Bradley et al. 1996). When we applied a mutation rate of 31.4% per million years, the estimated divergence times for the different lineages/clades detected ranged from 46,800 to 67,000 for the mtDNA-cr data, suggesting that their divergences occurred during the last glaciation. This obvious discrepancy can be solved by further calibration of the mutation rate of mtDNA in moose.

Demographic expansion of the Biebrza group of moose from the clade Central Europe deduced from the mismatch distribution suggests that it occurred soon after moose colonized Poland after the Last Glacial Maximum. It is
noteworthy that spatial expansion was much recent as our estimate from mtDNA-cr and its 95% CI suggests that it could have occurred in historical times.

Another noteworthy finding was the occurrence of very high levels of genetic differentiation among moose populations in Poland where the species reaches the natural western edge of its distribution. There are a few potential explanations for the high and significant differentiation of the population of moose. First, the studied moose populations could have experienced bottlenecks in the past that resulted in profound differences in haplotype frequencies among populations. Indeed, severe bottlenecks were documented during historical times in Poland (Brincken 1826), former East Prussia (Steinbach 2009), and even in Sweden (Charlier et al. 2008). Second, the gene flow among moose populations may be restricted (Charlier et al. 2008). This may be due to the moose dispersal pattern. Radio-tracking data indicate that both females and males are philopatric (Cederlund and Sand 1994).

High and significant pairwise $F_{ST}$ values between the moose populations studied as well as the estimates of migration parameter, $M$, deduced from population-specific $F_{ST}$ values seem to confirm limited female dispersal in moose, at least for the majority of populations. Third, Hallatschek et al. (2007) evidenced that range expansion in two dimensions results in an increase in the genetic differentiation among populations, as measured by $F_{ST}$. The colonization pattern based on moose dispersal from the east, successful reintroduction in Kampinos, and the presence of an autochthonous population in Biebrza, as well as remnants of a large population in East Prussia, could have resulted in spatial structure and the presence of sharp mtDNA haplotype frequency gradients after range expansion that occurred after the Second World War, e.g., about 70 years ago. This finding is, to our knowledge, the first evidence for the great and significant population structuring of an ungulate species that occurred in a recently recolonized area within less than ten decades. Indeed, SAMOVA and PCA showed that the genetic differentiation among the studied moose populations exhibited a clear spatial pattern. The relict, autochthonous Biebrza population was the most genetically divergent from all other ones. The second group was the Kampinos population that experienced a founder effect during the first years of reintroduction; three eastern moose populations in Poland that originated from moose immigrants from the east formed group 3, while the Srokowo and Gostynin-Wloclawek Forests populations seemed to be intermediate to the other populations on PCA. They could also possess some signs of introgression from East Prussia. These results are consistent with the proposed colonization pattern of Poland by moose after the Second World War (Gębczyńska and Raczyński 2004).

The relict Biebrza population is not only the most genetically different from the other populations but also, as our results revealed, is composed of three distinct subpopulations occurring in three basins. The presence of clearly differentiated subpopulations is most probably related to strong female philopatry. On the other hand, the high frequency of the haplotype H2 (mtDNA-cr) in the subpopulation from the upper basin clearly shows that its origin is from the neighboring Augustów population and that female dispersal between Biebrza and the neighboring Knysyn populations is limited.

In conclusion, this study emphasizes the existence of high levels of genetic differentiation among moose populations in Poland for mtDNA-cr, but not for Y-linked markers. It also suggests a variety of factors that may underlie this phenomenon, including bottlenecks, the presence of relict populations, reintroductions, limited female dispersal, as well as the colonization pattern. A complete elucidation of how these different factors define the patterns of genetic variability in moose will require more intensive sampling of animals from larger geographical areas and a wider range of nuclear markers.

Acknowledgments We are grateful to the Head of the Polish State National Forest Holding and the Biebrza National Park for kindly allowing us to collect moose stool samples from Poland and P. Rode for drawing the figures. The study was financed by the Ministry of Science and Higher Education, the University of Białystok (NN304 024134 and BST-117, respectively) for the Biebrza NP population, and by the National Fund for Environmental Protection and Water Management for the other populations (project no. 326/09/Wn50/NE-PR-IX/D).

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