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Genetic diversity and relatedness within packs in an intensely hunted population of wolves *Canis lupus*

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A population of grey wolves *Canis lupus* Linnaeus, 1758 inhabiting Białowieża Primeval Forest (BPF) on the Polish-Belarussian border has recovered after near extermination in the 1970s. Currently, it is intensively hunted in the Belarussian part of BPF and protected in the Polish part. We used a combination of molecular analysis, radiotracking, and field observation to study genetic diversity of the population after natural recolonisation and the consequences of heavy hunting for the genetic composition and social structure of wolf packs. Both microsatellite and mtDNA analyses revealed high genetic diversity. For 29 individuals and 20 microsatellite loci, the mean expected heterozygosity was 0.733. Four mtDNA haplotypes were found. Three of them had earlier been described from Europe. Their geographic distribution suggests that wolves recolonising BPF immigrated mainly from the north-east, and less effectively from the east and south-east. We traced the composition of 6 packs for a total of 26 pack-years. Packs were family units (a breeding pair with offspring) with occasional adoption of unrelated adult males, which occurred more frequently in packs living in the Belarussian part of the BPF, due to heavy hunting and poaching. Breeding pairs were half-sibs or unrelated wolves. Pair-bonds in the breeding pair lasted from 1 to 4 years and usually broke by the death of one or both mates. Successors of breeding females were their daughters, while a successor of a breeding male could be either his son or an alien wolf. As is evident from Białowieża's wolves, high genetic diversity may result from immigration of outside individuals, which are easily recruited to a heavily exploited local population.

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Introduction

After several centuries of retreat due to extermination by humans, European wolves *Canis lupus* Linnaeus, 1758 have recently been increasing in numbers and slowly recolonising parts of their former range (Promberger and Schröder 1992). The current situation of wolves, with isolated populations in Southern Europe and 'diluted' populations in regions with ongoing predator control, raises many questions about genetic variability and inbreeding depression of wolves (Ellegren 1999, Flagstad *et al*. 2003), their interbreeding with dogs (Lorenzini and Fico 1995, Randi *et al.* 2000, Andersone *et al.* 2002, Randi and Lucchini 2002), and the viability of small populations (Ciucci and Boitani 1991, Vila *et al.* 2003). Recent studies on genetic diversity of European wolves yielded mixed results. Ellegren *et al.* (1996) demonstrated that a small population of wild wolves in Sweden showed a low and still declining individual heterozygosity, and was monomorphic for a mtDNA control-region haplotype. Flagstad *et al.* (2003) showed that during the last two centuries approximately 40% of the microsatellite allele diversity and 30% of the heterozygosity had been lost. An isolated Italian wolf population showed a unique mtDNA haplotype and lower heterozygosity at microsatellite loci, when compared to other world populations (Randi *et al.* 2000, Lucchini *et al.* 2004). In a large-scale study, Vila *et al.* (1997) found 10 haplotypes of mtDNA in 13 European countries, a number well comparable to 11 haplotypes described from Asia (Tsuda *et al.* 1997, Vila *et al.* 1999) and 6 from North America (Lehman *et al.* 1991). This suggests that European wolves, though decimated in numbers and restrained to a small portion of their former range, were able to preserve a high degree of genetic polymorphism.

In this paper, we report on the genetic diversity and social structure of wolves inhabiting Białowieża Primeval Forest, a 1500-km^2 woodland located on the western border of the continuous geographic range of wolves in Europe. The local population has been suffering long-lasting disturbance by humans (intense hunting harvest and poaching) but survives due to the steady flow of immigrant wolves from other woodlands (Jędrzejewska *et al.* 1996). The aims of our 7-year study were to determine: (1) the genetic variability of the wolf population in terms of nuclear and mitochondrial DNA, (2) the relatedness of wolves belonging to the same and to different packs, and (3) the consequences of heavy hunting exploitation by humans for the genetic variability and social structure of wolf populations.

Material and methods

Sampled population and study area

In 1994–2000, we studied the wolf population inhabiting Białowieża Primeval Forest (BPF), which straddles the Polish-Belarussian border (52°45'N, 24°53'E). BPF contains one of the best preserved temperate woodland in the European lowlands. It covers about 1500 km^2 and adjoins other large woodlands to the north, east, and south-east (see Jędrzejewska and Jędrzejewski 1998, for more information on BPF). Wolves are permanently or ephemerally recorded in all the adjoining forest tracts. During the study, the density of the wolf population in BPF varied from 2 to 2.6 individuals/100 km^2 (numbers in winter; Okarma *et al*. 1998). In the 19th and 20th century, the population underwent three periods of severe control leading to temporary exterminations. The latest occurred in 1946–1960, and resulted in near absence of wolves from BPF in 1958–1972 (Jędrzejewska *et al.* 1996). Since the early 1970s, the population has been recovering mainly due to immigration of wolves. Since 1989, wolves have been protected in the Polish part of BPF (600 km^2), but they are still hunted in the Belarussian part (900 km^2), where 5–16 wolves are shot annually (10–64% of winter numbers) (Jędrzejewska and Jędrzejewski 1998).

Field data on wolf pack distribution and social system

Data on wolves were gathered in order to identify the number and locations of packs and their composition. In the Polish part of BPF, the main method was radiotracking. In 1994–1999, 12 wolves were live-trapped in nets or footsnare traps (see details in: Okarma and Jędrzejewski 1997, Jędrzejewski *et al*. 2000). Sex, approximate age, and reproductive status were determined for each wolf based on external features and tooth wear. This method allows for an accurate identification of age in juvenile (< 1 year) and subadult (1–2 years) wolves, and the accuracy declines with wolf age (to ± 2 years at the age of 6–8 yrs). Eleven wolves were equipped with radio-collars (Telonics Inc., AVM Instrument Company, Telemetry Systems, and Advanced Telemetry Systems) and radio-tracked for 1 to 38 months each. Over 40 000 locations were obtained. In addition, field observations on wolf pack composition were also gathered by snowtracking, stimulated howling, and recording any sightings of wolves (see Jędrzejewski *et al*. 2002). In 1994–2000, a total of 250 visual observations of wolves, 154 records of howling, and 894 observations of tracks were gathered.

In the Belarussian part, data on wolves were obtained from records of hunting efforts, field observations (snowtracking, casual sightings), and winter inventories of wolves. The majority of wolves were hunted with *fladry*. Wolves were localised by snowtracking and the area, where they were found (ca 1 km^2), and were surrounded with *fladry*, ie a rope with loosely hanging strips of bright-coloured, usually red, cloth. Wolves, which would not cross a *fladry* line, were then driven towards hunters stationed at the gap in *fladry* (see details in Jędrzejewska *et al.* 1996; Okarma and Jędrzejewski 1997). During such hunts, the whole pack was often targeted and its size and composition was noted. In total, from January 1995 till January 2000, hunters and game wardens conducted 43 hunts, and shot 62 wolves. Age (based on external appearance and tooth wear), sex, and reproductive status of shot wolves were determined. In addition, 31 field observations on wolves were gathered (tracks in snow, records of howling, sightings). Also, the data on wolf numbers from annual inventories of wolves conducted by game wardens in the Belarussian part of BPF were used as auxiliary information for the mapping of wolf packs. Inventories were carried out during winter (December to February). After new snow fall, snowtracking along all accessible forest compartment lines (the grid of 1066 × 1066 m = 1 × 1 verst) was conducted twice, on two consecutive days to get a reliable estimate. Tracks of wolves crossing the lines, their direction and number of individuals were noted. Then, all tracks were mapped and the locations of wolf daily resting sites were determined (ie forest compartment, where a track went into but not out of it). The inventory yielded the numbers of wolves in each of 10 forest districts.

Analyses of mitochondrial and nuclear DNA

In the Polish part of BPF, material for DNA analyses was collected from live-trapped wolves (n = 12), wolves found dead or poached (n = 3), and wolves taken as pups from a den by poachers and later bred in captivity (n = 3) (see the list in Appendix I). Samples from the Belarussian part (n = 17) came from wolves shot by hunters (Appendix I). In total, 35 specimens (17 females, 18 males) from BPF or close vicinities were analysed. Samples from live wolves were collected as hairs with roots and those from dead animals as soft tissue (heart, liver, muscle) and hairs with roots. Samples were kept frozen (-20^{o} to -80^{o}C) until they were analysed.

DNA was extracted using the standard organic method (Sambrook *et al.* 1989). Samples were subjected to overnight digestion with proteinase K, followed by double phenol: chloroform: isoamyl alcohol extraction. Samples with a small amount of genetic material were additionally concentrated on Microcon 100 (Millipore, USA) concentrators. Mitochondrial DNA extraction was successful for 34 samples, and nuclear DNA extraction for 29 samples. Amplification of 300bp-long, highly polymorphic mtDNA fragment of the HV1 region was done using primer sequences described by Savolainen *et al*. (1997). The PCR reaction mixture was made up of 1U Taq polymerase, 200 µM dNTP, 2.0 µl 10 × concentrated PCR buffer, 1.5 mM $MgCl_2$ (all from Promega, Madison, USA), 0.1 mM of primers and usually 5 µl of DNA for 20 µl reactions. Amplifications were performed in a Perkin Elmer 9700 thermocycler. The reaction conditions were as follows: 2 min at 94°C – initial denaturation, 30 or 36 cycles of 20 s at 94°C, 30 s at 69°C, 40 s at 72°C, and the final elongation step for 10 min at 72°C. Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen, USA). Sequencing reactions were performed in a 9700 GeneAmp Perkin Elmer Thermal Cycler using dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Warrington, UK). Detection of sequencing reaction products was carried out on ABI 310 genetic analyser. The results of sequencing were analysed with ABI Prism DNA Sequencing Analysis software, version 3.0, and comparisons were performed using Sequence Navigator, version 2.0. Positive and negative controls were added at each stage of analysis.

The analysis of nuclear DNA was based on a microsatellite marker set of 20 di- and tetranucleotides. They were chosen because of their polymorphism among domestic dogs and other wolf-like canids. The primer sequences for the following dinucleotide markers stem from Fredholm and Wintero (1995): CPH02, CPH03, CPH04, CPH06, CPH07, CPH08, and CPH17. The primer sequences for the following tetranucleotide markers stem from Francisco *et al*. (1996): 2001, 2010, 2016, 2054, 2097, 2109, 2137, 2140, 2161, 2164, 2168, 2175, and 2201. Multiplex-PCR was carried out in 15 µl reactions using approximately 100 ng genomic DNA in 1.5 mM $MgCl_2$ (Sigma), 200 mM dNTP (Peqlab), 1x buffer (Sigma), and 0.5U Taq polymerase (Sigma). The forward primer of each microsatellite marker was synthesised with an additional tail of M13MP18 phage ($^{5'}$CGT TGT AAA ACG ACG GCC AGT$^{3'}$). The complementary primer to this tail was labelled with fluorescent dye, either TET, FAM or HEX. Those Multiplex-Amplifications were achieved with the following conditions. After a first step of 94°C and 4 min, 10 cycles followed with denaturation 94°C, 1 min; annealing 60°C, 1 min, and extension 72°C, 1 min. For the next 30 cycles, annealing temperature was changed to 55°C, and the whole reaction ended with a last extension step of 72°C, 7 min. Fragment analysis was done with a Sequencer ABI 310 (Perkin Elmer) using an internal TAMRA--labelled standard.

The program GenePop 3.3 (Raymond and Rousset 1995) was used to calculate expected heterozygosity, to check for heterozygosity excess in the population, to test population differentiation and to check the correlation between genetic and geographic distance among packs (Mantel's test). Deviation from Hardy-Weinberg equilibrium (heterozygosity excess) was checked with an exact test using a Markov chain algorithm (Guo and Thompson 1992). The analysis of molecular variance (AMOVA) was done using the Arlequin software (Schneider *et al*. 2000). The assignment test and detection of first generation migrants were performed using the program GeneClass 2 (Piry *et al*. 2004). Both tests were performed using Rannala and Mountain's (1997) Bayesian method and Monte-Carlo resampling algorithm of Paetkau *et al*. (2004) with 1000 simulated individuals and type I error 0.01. In the detection of migrants we used L_{home}/L_{max} likelihood computation, which is the ratio of the likelihood computed from the population, where the individual was sampled, to the highest likelihood

value among all populations including the population where the individual was sampled (Paetkau *et al*. 2004). Relatedness within packs and pairwise relatedness were calculated using the program Relatedness 5.0 (Queller and Goodnight 1989). The parentage analysis was performed using the program Cervus 2.0 (Marshall *et al.* 1998); standard parameters were used in the simulation (10 000 cycles, 10 candidate parents, rate of typing error 0.01). The results of parentage assignment (see Appendix II) were verified by checking values of pairwise relatedness, concordance of mtDNA haplotype of the offspring and potential mother, and all other information available from radio-telemetry (sex, age, belonging to the same pack).

Results

Genetic diversity of wolf population

In 1995–2000, seven to nine packs of wolves inhabited BPF. Genetic data were obtained from 8 of them and from additional 2 packs inhabiting the adjoining forests (Fig. 1). Among the analysed wolves, four haplotypes of mtDNA were found (accession numbers in GenBank: H1 – AF344299, H2 – AF344300, H3 – AF344301,

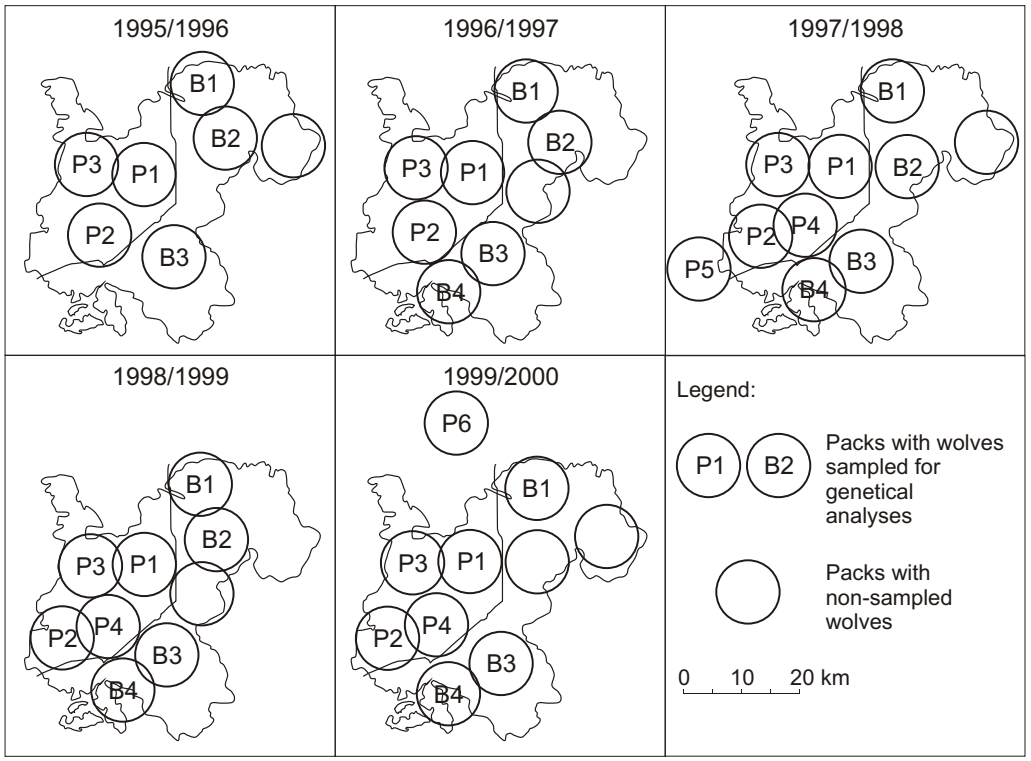

Fig. 1. Schematic spatial distribution of wolf *Canis lupus* packs in Białowieża Primeval Forest (Poland and Belarus) in the autumn-winter seasons of 1995/1996–2000. P – packs in the Polish part of BPF, B – packs in the Belarussian part. Circles approximate core areas of wolf territories.

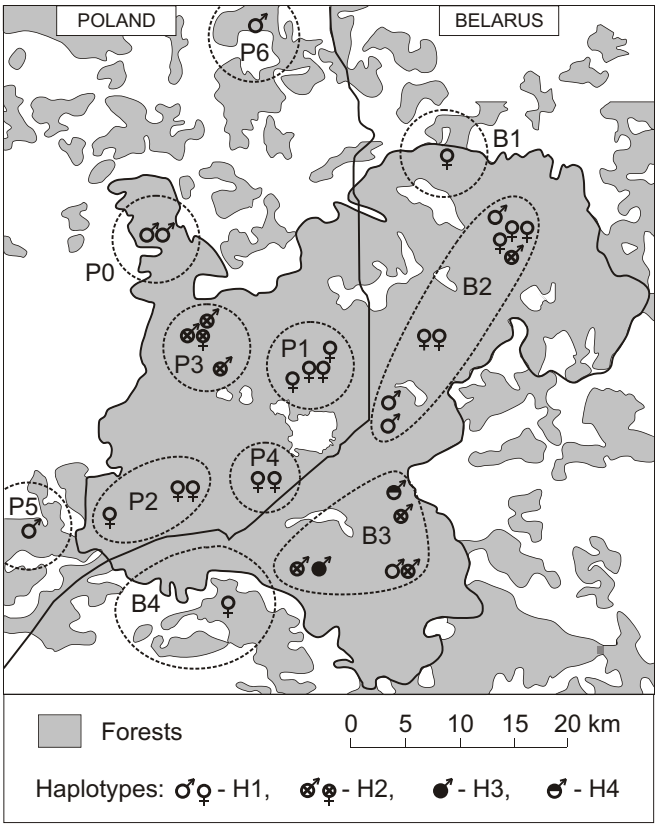

Fig. 2. Spatial distribution of wolves possessing four different haplotypes of mtDNA in BPF. Symbols are located in places, where wolves were shot (Belarussian part) or in the core areas of wolf territories (Polish part). Dashed lines encircle wolves belonging to one pack (pack symbols as in Fig. 1).

H4 – AF344302). Differences occurred in nine nucleotide positions. Pair-wise comparisons of the haplotypes revealed 2.7% maximum sequence divergence between them (seven nucleotide substitutions). It is worth noting that there was only one substitution between haplotypes H2 and H4. Among 35 wolves, haplotype H1 was the most common (25 individuals, 71%). H2 was found in 8 wolves (23%), H3 and H4 in one wolf each (3% each). The two rarest haplotypes were represented only by males. Of seven packs sampled in the Polish part, six had H1 and one had H2 haplotypes. In four packs with two or more wolves studied, all wolves in a pack shared the same haplotype (Fig. 2). The population sample from Belarus was more variable. In four sampled packs, all four haplotypes were found. Two packs with two or more wolves studied were comprised of wolves with two (pack B2) or four (pack B3) haplotypes (Fig. 2). Frequency of haplotypes did not differ significantly between the Polish and the Belarussian parts of BPF (*G*-test: $G = 0.992$, df = 3, $p > 0.5$).

Table 1. Expected unbiased heterozygosity (Nei 1978) and number of alleles per locus in wolves *Canis lupus* from Białowieża Primeval Forest.

Marker	Hetero-zygosity	Number of alleles
Tetranucleotyde repeats		
2001	0.683	5
2010	0.708	5
2016	0.785	8
2054	0.742	8
2097	0.534	5
2109	0.507	3
2137	0.723	9
2140	0.692	5
2161	0.807	7
2164	0.899	12
2168	0.810	10
2175	0.872	10
2201	0.835	8
Mean (SE)	0.738 (0.033)	7.31 (0.72)
Dinucleotyde repeats		
CPH02	0.728	6
CPH03	0.800	8
CPH04	0.711	7
CPH06	0.690	6
CPH07	0.753	5
CPH08	0.697	5
CPH17	0.681	5
Mean (SE)	0.723 (0.273)	6.00 (0.44)
Mean for all (SE)	0.733 (0.022)	6.85 (0.50)

The mean number of alleles was 6.85 over all microsatellite loci, 6.0 over dinucleotide loci, and 7.31 over tetranucleotide loci (Table 1). The mean expected heterozygosity was 0.733 for all microsatellite loci, 0.723 and 0.738 for dinucleotide and tetranucleotide loci, respectively (Table 1). Hardy-Weinberg exact test implemented in GenePop program indicated heterozygosity excess (the probability value associated with H_0: $p = 0.110$, the standard error of this estimate: SE = 0.005).

Genetic composition and social structure of packs

The relatedness among all possible pairs of wolves (all individuals were weighted equally) varied from –0.600 to 0.835, on average 0.004 (SE = 0.011, $n = 405$ pairs). The relatedness among wolves belonging to the same pack (mean 0.234, SE = 0.031, $n = 67$) was significantly higher than those among wolves from different packs (mean –0.042, SE = 0.011, $n = 338$; $t = 10.042$, $p < 0.0005$). Furthermore, females within a pack were more closely related to each other (mean 0.324, SE = 0.042) than were males belonging to the same packs (mean 0.190, SE = 0.057), though the difference was not significant ($t = 1.554$, $p = 0.128$).

Spatial distances between the centres of territories of all possible pairs of wolf packs ranged from 10 to 55 km. At such a local scale, the genetic distance (measured as F_{ST}) among wolves from different packs was not related to the spatial distance among them (Mantel test, $p = 0.97$). Two tests were performed to check whether Polish and Belarussian packs are genetically distinct. The analysis of molecular variance did not show any population genetic structure: 98% of variation was explained by within population variation and the fixation index was low ($F_{ST} = 0.024$, $p = 0.025$). The exact test of population differentiation (Raymond and Rousset 1995) did not show that the population was genetically differentiated, either ($p = 1.0$, 6000 Markov steps done).

Assignment test and detection of first generation migrants were performed only for four packs with more than two individuals genotyped, because of increased probability of assignment to the groups with small number of individuals. All individuals from Polish packs (P1 and P3) were assigned (with $p > 0.95$) to their own packs. In case of Belarussian pack B2, 7 individuals were assigned (with $p > 0.94$) to their own pack, while 3 individuals were not unequivocally assigned to any pack. Female 17 was assigned to the pack B2 with a probability 0.802 and to the pack P3 with a probability 0.519. Her assignment probabilities to other packs were low ($p < 0.16$). According to parentage analysis (see below), the mother of both these females was female 25 from the pack B2, but their fathers remained unknown and could have been immigrants from other packs. Male 12 was assigned to the pack B2 with a probability 0.167, and his assignment probabilities to other packs were very low ($p < 0.03$). This male was identified as a first generation migrant and the pack P1 was indicated as the most likely population of his origin. This result was confirmed by pair-wise relatedness computations: male 12 was not related to individuals from the pack B2, and was most closely related to female 1 from the pack P1. However, the assignment test did not confirm the origin of this individual from the pack P1. Also, the mtDNA haplotype of this individual (H2) was different from that of individuals from the pack P1 (H1). So it is also possible that the male 12 was an immigrant from another, unknown population.

In the case of the Belarussian pack B3, 4 individuals were assigned (with $p > 0.97$) to their own pack, while male 29 was not unequivocally assigned to any pack. He was assigned to the pack B3 with a probability 0.735, and to the pack P3 with a probability 0.650. This male was identified as a first generation migrant and the pack P3 was indicated as the most likely population of his origin. This result was also confirmed by pair-wise relatedness computations: M29 was not related to individuals from the pack B3, and was most closely related ($R = 0.60$) to M9 from the pack P3. However, as the male 12 was the sole carrier of H3 haplotype, it is also possible that he was an immigrant from another, unknown population.

The most probable genealogies in 6 packs were traced in 2 to 7 consecutive years (a total of 26 pack-years; P0 not included), based on the exclusion power of a test implemented in a program Cervus 2.0, the values of pair-wise relatedness, the concordance of mtDNA haplotype between mother and offspring, all other information available from radiotelemetry in the Polish part (sex, age, belonging to the same pack), and data from hunting records in the Belarussian part (wolves shot together in one place during a hunt). Results are presented in Fig. 3. The core of the pack was always a breeding pair and their offspring of the current year. In several cases, the presence of the former-year(s) offspring in a pack was also recorded. In two packs, non-breeding adult males unrelated to the breeding pair and their offspring were found (M12 in pack B2, and M29 in pack B3); these two individuals have been identified as first generation migrants. The relatedness of a

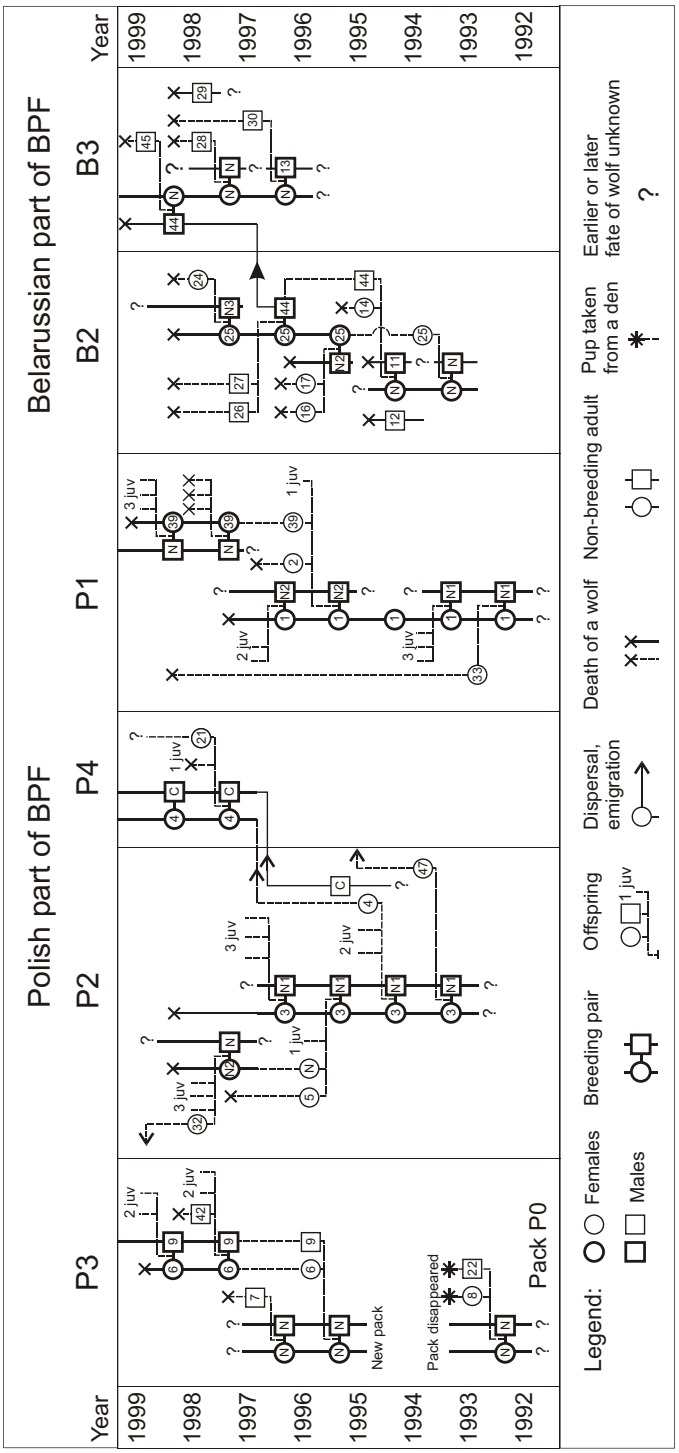

Fig. 3. Pack composition and family relationships in seven packs of wolves. N – wolves unmarked and not sampled for genetic analysis. C – unmarked, but regularly observed, visually distinctive male (black-coloured). Data prior to 1994 show only those wolves that were studied in 1994–2000 and that had been born earlier. Wolf numbers as in Appendix I, parentage assignment as in Appendix II.

breeding pair was examined in one case, only. In pack B2, F25 mated with her half-brother M44 in 1997 (Fig. 3). As suggested by observations and records on their pack's history, M6 and F9, which formed a breeding pair in pack P3, might also have been sibs as well. In both cases, formation of a pair was preceded by deaths or disappearance of the former breeding individual(s).

High mortality of wolves due to hunting in the Belarussian part and poaching in the Polish part was the main reason of frequent changes in the composition of mating pairs. In pack P1, F1 mated with 2 males during 5 years (Fig. 3). In pack B2, F25 mated with 3 males in 3 consecutive years. During his 5.5-year life, M44 mated in two packs, his natal one (B2) and a new one (B3). In the six packs, 10 breeding females were recorded. They held their dominating position and attempted breeding in at least 1 to 5 years, on average 2.6 years (SD 1.2). Five females were shot or poached. In two cases, some other members of a pack had been shot, and the breeding female was no longer observed in that pack though her fate remained unknown. Only one old female (F3 in pack P2), after having reared at least 4 consecutive litters, ceased breeding while still in a pack. Then, she was recorded moving alone on the verges of the pack's territory and eventually was found dead one year later.

The high turnover of the breeding females raises a question of their succession. In four cases, when the breeding females were killed or otherwise lost their position, their daughters became successors (packs P1 and B2, as revealed by microsatellite analysis, radiotracking, observations, and packs P2 and P3, as suggested by radiotracking data). We recorded at least 13 males, which held their breeding position in a pack for a minimum of 1 to 4 years, on average 1.8 years (SD 0.8). Three of them were killed, one (M44) emigrated from his natal pack after having bred there for one year (Fig. 3). The persistence of pair-bonds estimated for 14 breeding pairs of wolves varied from 1 to at least 4 years, on average 1.8 (SD 0.8). In all cases except one, the cause of disruption was the death of one or both mates. Three wolves dispersed from their natal packs but remained in BPF. Male 44 shifted from pack B2 to B3 (34 km). F4 left pack P2 (together with male C of unknown origin) and founded a new pack P4 nearby (6 km; see Jędrzejewski *et al.* 2004). Furthermore, parentage assignment suggests that M12 recorded in pack B2 was born in pack P2. At least two cases of dispersal beyond BPF were recorded based on radiotracking (F47 and F32, both from pack P2).

In total, during 27 pack-years (pack P0 included) at least 52 young wolves born in BPF were recorded and the fates of 26 young was known. Among those 26 wolves, 15 (29% of all born) were killed by humans or died from other causes before having a chance to reproduce, 2 continued to live in captivity for some years, 7 wolves survived and bred in the study area, and 2 emigrated beyond BPF. The minimal fraction of native wolves that entered breeding would be 13%, if estimated on a more conservative assumption that they were 7 out of all 52 locally-born wolves.

Discussion

Regarding the recent history of extermination in the 1970s, genetic variability of Białowieża's wolves appeared very high in comparison to North American and Swedish populations (Table 2). High genetic diversity of wolves in BPF was also proved by our revealing of four mtDNA haplotypes. So far, 34 maternal lineages were described in the world populations of the grey wolf (review in Vila *et al.* 1999). It must be noted, however, that a major part of the wolf's geographic range remains unstudied (eg. Eastern Europe, Russia, Caucasus Mountains, Kazakhstan). Among four haplotypes found in Białowieża, three were identical with some of those already known (Ellegren *et al.* 1996, Vila *et al.* 1999, Randi *et al.* 2000) and they have an interesting geographic distribution. H1 was reported from north-eastern Europe but not from southern part of the continent, H3 stretched in south-east Europe and the Near East (Bulgaria, Saudi Arabia), and H4 was reported from Russia and Romania (Fig. 4). In the 1970s, it also occurred in Sweden, but now it is most probably extinct there (Ellegren *et al.* 1996). Moreover, H4 was found in several dog breeds (Vila *et al.* 1997; W. Branicki, unpubl. data). Haplotype H2, which was found in 26% of the studied wolves in BPF, is new; so far it has been reported from neither Eurasia nor America.

The geographic distribution of haplotypes H1, H3, and H4, described above and confirmed also by a large-scale study in Eastern Europe (W. Jędrzejewski, M. Pilot and co-workers, unpubl. data), suggests that wolves recolonising BPF immigrated mainly from the north-east and, less effectively, from the east and south-east. BPF is located near the watershed of the Vistula, Nemen, and Dnestr rivers, and it is connected via wide forested corridors with other large forests and wilderness areas in Belarus, Lithuania, Russia, Ukraine, and eastern Poland (Faliński 1986). From the standpoint of nature conservation, it is highly recommended to reconstruct forest corridors from BPF towards the west, to enable migrations of wolves and other species to woodlands and forests of Central and Western Europe.

Forbes and Boyd (1996), who studied wolves naturally colonising Glacier National Park and surrounding lands (USA), found high genetic variability in the

Table 2. Genetic diversity of wolves in BPF (Poland – Belarus) compared to other Holarctic populations (in all cited studies: analysis of microsatellite set composed of 7–12 dinucleotids).

Country	Number of microsatellite loci examined	Mean number of alleles per locus	Mean heterozygosity	Source
Sweden	12	2.8	0.470	Ellegren *et al*. (1996)
Canada (5 regions)	10	3.4–6.4	0.566–0.741	Roy *et al.* (1994)
USA (2 regions)	10	4.1–6.3	0.581–0.686	"
Poland – Belarus	7	6.0	0.723	This study

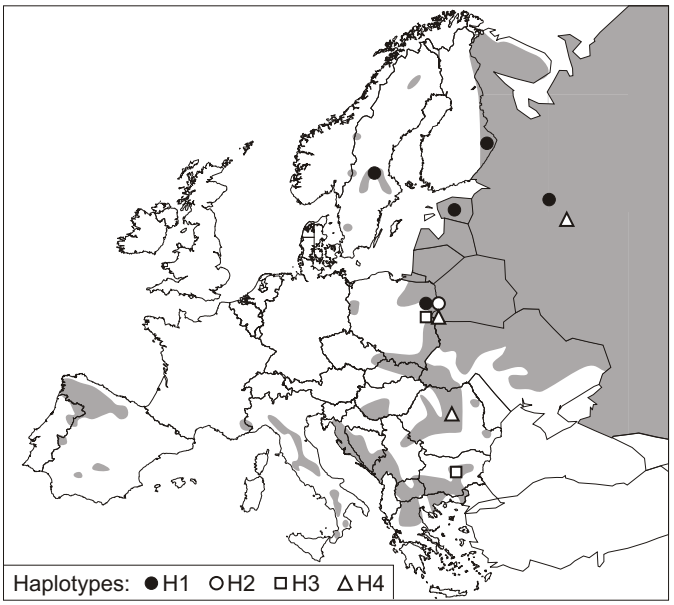

Fig. 4. Contemporary geographic distribution of 4 haplotypes of mtDNA recorded in wolves from BPF. Sources: Vila *et al.* (1997), Ellegren *et al.* (1996), and this paper. Symbols denote countries of the origin of studied wolves but not the detailed localities. Haplotype H3 was also recorded from Saudi Arabia. Range of wolves in Europe (shaded) after Promberger and Schröder (1992), modified.

colonising population founded by multiple immigrations of unrelated wolves from Canada. In European wolves, as many as 7 haplotypes were found among 26 individuals from Bulgaria and 4 haplotypes among 7 wolves from Greece (Vila *et al.* 1999, Randi *et al.* 2000), proving high genetic variability in outbred populations. In contrast, in the isolated or nearly isolated wolf populations, such as those in Italy and Sweden, a notable loss of genetic variability was recorded (Ellegren *et al.* 1996; Randi *et al.* 2000, Flagstad *et al.* 2003, Lucchini *et al.* 2004).

In North America, the genetic relationships of wolves within and among packs were studied by Lehman *et al.* (1992) and Meier *et al.* (1995). Their findings confirmed earlier observations that wolf packs were family units: the genetic similarity was significantly higher among wolves from the same packs than among packs. However, Lehman *et al.* (1992) and Meier *et al.* (1995) also recorded cases of adoption of strange wolves into packs, short-distance dispersal within a local population, dissolution, and splitting of packs. Meier *et al.* (1995) concluded that even in the absence of significant human disturbance, the longevity of packs and their stability were less than previously suggested, and the genetic distinctions between packs tended to be blurred by shifts of individuals among packs. Our study conforms to those findings. The high turnover of individuals in and among the packs was manifested in Białowieża, as the population was heavily

exploited by humans and nearly all mortality of wolves was caused by human hunters and poachers. Importantly, the most intense hunting season in BPF lasts from November till March, eg prior to, during, and soon after the wolf mating season (January–February). This resulted in short persistence of pair-bonds, because one or both breeding wolves were often killed during the winter hunting season, and decreased relatedness among pack members, as litters born to a pack in consecutive years were often half-sibs and not full sibs. Packs reduced by hunting to few wolves or merely a pair are probably more tolerant to adopting strange wolves in order to survive and rear young. This study showed that the two well-sampled Belarussian packs adopted unrelated single wolves that might be long-distance dispersers. Also, in the North American wolves, Fritts and Mech (1981) observed that a dead parent was replaced by an outside wolf.

The loss of genetic variability is a dominant concern of conservation genetics (Caughley 1996). Our study was evidence for the opposite situation, ie high genetic polymorphism and heterozygosity excess in the wolf population with a social structure disturbed by intensive hunting. The most probable explanation for this result is a high immigration rate. Immigrant wolves may be more easily recruited to a population, which steadily suffers from heavy exploitation.

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Appendix I. Data on age, sex, and fates of wolves *Canis lupus* analysed in this study. Symbols of packs as in Fig. 1. Genetic samples: mtDNA haplotypes, H1 to H4, are written wherever known from mtDNA study. Symbols in parentheses – haplotype deduced from close relatedness in the maternal lineage as revealed by nuclear DNA (microsatellite) analysis. Wolves with nuclear DNA tested are marked with "+".

Wolf no.	Pack	Sex	Year of birth	Month and year of death	Timespan of radiotracking	Genetic samples		Cause of death, other fate, notes
						mtDNA	nuclear DNA	
1	2	3	4	5	6	7	8	9
1	P1	F	1989–1991	Nov 1997	Mar 1995 – Nov 1997	H1	+	Poached with snare
2	P1	F	1996	Oct 1997	Dec 1996 – Oct 1997	H1	+	Poached with snare
3	P2	F	1988–1992	Jul 1998	Jan 1995 – Jul 1998	H1	+	Died
4	P2, P4	F	1995		Nov 1997 – Dec 1998	H1		Born in P2, in 1997 left the pack together with a black-coloured male (C) from P2 and founded a new pack P4; radiotracking discontinued due to collar failure
5	P2	F	1995	Feb 1998	Dec 1997	H1	+	Shot in the Belarussian part of BPF
6	P3	F	1996	Jun 1999	Jan 1998 – Jun 1999	H2		Poached (shot)
7	P3	M	1997	Feb 1998	Oct 1997 – Feb 1998	H2	+	Poached with snare
8	P0	M	1993		–	H1		Taken from den as pup (by a poacher), together with M22 and kept in captivity; in 1994–1995, pack P0 disappeared and was replaced by a new pack P3
9	P3	M	1996		Jan 1998 – Sep 1999	H2	+	Radiotracking ended due to collar failure
11	B2	M	1989–1992	Dec 1995	–	H1	+	Shot
12	B2	M	1989–1993	Dec 1995	–	H2	+	Shot together with F14
13	B3	M	1992–1994	Jan 1997	–	H4	+	Shot
14	B2	F	1995	Dec 1995	–	H1	+	Shot together with M12
15	B1	F	1991–1993	Dec 1995	–	H1	+	Shot
16	B2	F	1996	Jan 1997	–	H1	+	Shot together with F17
17	B2	F	1996	Jan 1997	–	H1	+	Shot together with F16
21	P4	F	1998		–	(H1)	+	Live-trapped in Sep 1998 but not collared
22	P0	M	1993		–	H1	+	Taken from den as pup (by a poacher), together with M8, kept in captivity

Appendix I – continued.

1	2	3	4	5	6	7	8	9
23	P5	M	1992–1996	Sep 1998	–	H1		Found dead, cause of death unknown
24	B2	F	1998	Dec 1998	–	H1	+	Shot together with F25
25	B2	F	1994	Dec 1998	–	H1	+	Shot together with F24
26	B2	M	1997	Dec 1998	–	H1	+	Shot
27	B2	M	1997	Feb 1999	–	H1	+	Shot
28	B3	M	1998	Jan 1999	–	H2	+	Shot together with M29 and M30
29	B3	M	1993–1994	Jan 1999	–	H3	+	Shot together with M28 and M30
30	B3	M	1997	Jan 1999	–	H2	+	Shot together with M28 and M29
31	B4	F	1997–1998	Feb 1999	–	H1	+	Shot
32	P2	F	1998		Oct 1998 – Jan 1999	H1		Radiotracking discontinued (lost contact); premature dispersal after her mother was shot in the Belarussian part in Nov 1998
33	P1	F	1993	Jan 1999	Mar 1994 – Feb 1995	H1	+	Poached (shot); radiotracking discontinued due to collar failure
39	P1	F	1996	Sep 1999	Jan 1998 – Sep 1999	H1		Found dead in her territory, cause unknown
41	?	M	1999	Oct 1999	–	H1	+	Found dead from injuries (bites) in the territory of pack P1
42	P3	M	1998	Mar 1999	–	H2	+	Found dead in his natal territory, cause unknown
44	B3, B4	M	1995	Nov 1999	–	H1	+	Shot together with M45
45	B3	M	1999	Nov 1999	–	H2	+	Shot together with M44
46	P6	M	1999		–	H1	+	Taken from a den as pup (by a poacher), kept in captivity
47	P2	F	1993		Dec 1995 – Apr 1996			Live-trapped and radio-collared as subadult in pack P2, dispersed beyond BPF

Appendix II. Results of parentage assignment. All candidate parents with positive LOD scores, whose parentage can not be excluded because of age (ie they were older then putative offspring), are enclosed. We accepted parent-offspring relationship with one loci mismatched, that may result from genotyping errors, if at least 10 loci were compared. Parent-offspring relationship suggested by the analysis of smaller number of loci was accepted only if confirmed by the radiotracking data. Eventually assigned parent-offspring pairs are in bold. See Notes below this table for further details.

Offspring (O)		Probability of nonexclusion	Candidate Parent (CP)		Number of loci in O-CP pair		LOD score	Delta
Pack, wolf	N loci typed		Pack, wolf	N loci typed	N loci compared	N loci mismatched		
1	2	3	4	5	6	7	8	9
B2 M12	19	0	P2 F3	6	6	1	0.442	0.442
B2 F14	20	0.0001	**B2 M11**	19	19	0	4.222	2.718
"	"	"	B2 M44	15	15	3	1.503	0
B2 F16	20	0.0004	**B2 F25**	17	17	1	3.277	0
B2 F17	20	0.0001	**B2 F25**	17	17	1	2.609	0
B2 F24	16	0.0014	B2 M27	18	16	0	6.069	3.960
B2 F24	"	"	**B2 F25**	17	15	0	2.109	0
B2 F25	17	0.0051	**B2 M11**	19	16	1	0.389	0
B2 M26	15	0.0033	**B2 F25**	17	13	0	6.801	4.377
"	"	"	B2 F16	20	15	1	2.424	0
B2 M26	"	"	**B2 M44**	15	13	1	2.334	0
"	"	"	B2 M27	18	13	2	1.952	0
B2 M27	18	0.0005	**B2 M44**	15	13	0	5.118	0
B2 M27	"	"	**B2 F25**	17	17	1	3.402	0
"	"	"	B2 M26	15	13	2	1.952	0
"	"	"	P3 M09	6	6	1	0.434	0
"	"	"	B3 M29	10	9	2	0.228	0
B2 M44	15	0.0228	B3 M29	10	10	0	3.123	0
B2 M44	"	"	**B2 M11**	19	14	0	1.426	0
B3 M28	14	0.0010	B3 M30	15	13	4	1.481	0.844
B3 M28	"	"	B3 M13*	20	14	3	0.637	0
"	"	"	P0 M22	18	14	4	0.294	0
B3 M30	15	0.0002	**B3 M13**	20	15	1	2.815	1.334
B3 M45	10	0.0426	P2 F3	6	4	0	1.826	0.109
B3 M45	"	"	**B2 M44**	15	10	1	1.717	0
"	"	"	P4 F21	19	9	0	1.636	0
"	"	"	P0 M22	18	9	0	1.315	0
"	"	"	B3 M30	15	9	1	1.122	0
"	"	"	B3 M13	20	10	2	0.366	0
"	"	"	B3 M28	14	8	0	0.141	0
B4 F31	20	0	P3 M9	6	6	2	0.437	0.437
P1 F2	20	0.0004	**P1 F1**	18	18	0	5.029	3.783
"	"	"	P1 F33	18	18	2	1.245	0
P1 F33	18	0.0003	**P1 F1**	18	18	0	5.007	3.762
"	"	"	B1 F15	20	18	4	1.128	0

Appendix II – continued.

1	2	3	4	5	6	7	8	9
P2 F3	6	0.0629	B2 M12	19	6	1	0.442	0
P2 F5	17	0.0003	**P2 F3**	6	6	0	1.571	1.571
P3 M7	11	0.0014	B2 F17	20	11	3	1.577	0
P3 M9	6	0.126	B2 M11	19	5	0	2.652	0.904
"	"	"	B3 M29	10	5	1	1.748	0
"	"	"	B2 M44	15	6	0	0.948	0
P3 M41	15	0.0003	P3 M7	11	9	2	1.832	0.946
"	"	"	P3 M42	8	8	1	0.304	0
P3 M42	8	0.0154	**P3 M9**	6	2	0	1.111	0
"	"	"	B3 M29	10	7	1	0.754	0
"	"	"	P1 F2	20	8	2	0.384	0
P4 F21	19	0	P2 F3**	6	6	0	0.915	0

*Possible grandson (M28) – grandparent (M13) relationship.
**Granddaughter (F21) – grandmother (F3) relationship.

Notes:

Pack P1. F1 (a breeding female as evidenced by radiotracking) was the mother of F33 and F2. These two females, however, would have different fathers. Moreover, F39, radiotracked as adult in the pack P1, was most probably a pup from the same litter as F2.

Pack P2. F3 was assigned as the mother of F5. Only 6 loci could be compared between these two females, but we accepted this relationship, because the radiotracking data confirmed it. Long-term radiotracking of five female wolves and numerous visual observations of the pack members indicated that F3 was also a mother of F4, and a grandmother of F32. In the winter of 1997/1998, a pair of wolves (F4 and male C) left their original pack P2 and founded a new pack P4 (see Jędrzejewski *et al.* 2004). They were probably parents of F21, live-trapped as a 5-month-old pup in the core area of their territory in 1998. Indeed, according to molecular data, F3 could be a grandmother of F21.

Pack P3. Based on the radiotracking data, M9 and F6 were most likely parents of M42. M9 is also the most probable father of M42 according to LOD score (1.11) and relatedness coefficient (0.77), however, only 2 loci could be compared.

Pack B2. M11 was the most probable father of F14 (LOD = 4.22, R = 0.53). M29 was the most probable father of M44 (10 loci compared, LOD = 3.12, R = 0.59) and the second probable father was M11 (14 loci compared, LOD = 1.43, R = 0.45). However, because of the same age and the high relatedness of F14 and M44 (R = 0.51), they were probably full sibs from one litter and their father was M11 (M29 is not closely related to F14 and M11). F14 and M44 were probably half sibs of F25 (R = 0.28 and R = 0.20, respectively). M11 was the most probable father of F25 (LOD = 2.11), but relatedness coefficient of F25 and M11 (R = 0.28) did not confirm this interpretation; it is much lower than that of F14 and M11 (0.53) and M44 and M11 (0.45). The most plausible explanation is that F25, F14 and M44 had the same mother, and a father of F25 was a brother or a son of M11. M11 was also the most probable father of M9 from a pack P3 (LOD = 2.65), however, only 6 loci were compared. More importantly, M11 was shot one year before the most probable birth time of M9. Thus, we did not accept this assignment.

F25 was the most probable mother of F16 and F17. Shot together in one pack, F16 and F17 were of the same age, so we assumed that F16 and F17 were full sibs from the same litter, and the microsatellite profile did confirm this. F25 was also the most probable mother of M26 and M27, who were shot in one pack at the same age. According to LOD scores, the most probable father of M26 was his older brother, M16 (15 loci compared, LOD = 2.42, R = 0.40) and the second most probable father was M44 (13 loci compared, LOD = 2.33, R = 0.39). However, M16 could not have been a father of

M26, because he was only one year older. The most probable father of M27 was M44 (13 loci compared, LOD = 5.12, R = 0.71). So we accepted that both M26 and M27 were offspring of F25 and M44. The high relatedness coefficients of M44 and M27 (R = 0.71) and of F25 and M26 (R = 0.84) can be explained as a result of mating between half sibs (F25 and M44). F24, shot in a hunt together with F25, was most probably a daughter of F25 (15 loci compared, LOD = 2.11, R = 0.44). According to LOD score, F24 might be a daughter of M27 (16 loci compared, LOD = 6.07). This is, however, impossible because M27 was only one year older than F24. The high LOD score and the high relatedness coefficient of F24 and F27 (R = 0.63) can result from inbreeding. F24 is not a daughter of M44, but she might have been a daughter of an unknown individual closely related to M44 (his brother or son).

Pack B3. M13 was the most probable father of M30 and M28. However, in case of M13 and M28, 3 from 14 compared alleles are not concordant, and the relatedness coefficient (R = 0.21) is too small to be a father-son relationship. More probable is that M28 was a son of a close relative of M13 (a brother or a son) and that he had the same mother as M30. M44 was the most probable father of M45. These two males were shot together in pack B3, one year after pack B2, the original pack of M44, was eradicated by hunters. None of the putative mothers (F3, F21, F22) had mtDNA haplotype concordant with H2 haplotype of M45. All the wolves M30, M28, and M45 could have stemmed from the same, unknown mother that had the H2 haplotype.