

## **Annex 9**

### **Starting point assessment of the space use patterns and genetic structure of bison population (before implementing conservation activities) – associated actions – D3,D4,D5 actions (MIL2)**

#### **Number and distribution of bison**

Based on regular survey conducted at feeding sites and registration of all bison sightings, population size in Białowieża Forest was estimated on 402 bison in winter 2006/2007, 439 in 2007/2008, 456 in 2008/2009 and 451 in 2009/2010 (Table 1). During 4 years of the project population increase rate was 13.6% in comparison to bison number in 2005 before the project. The growth was mainly recorded among females and subadult individuals. In winter, bison gathered at feeding sites creating 5 large winter aggregations. Additionally, some number of bison roamed out of feeding sites, usually in the mosaic of meadow and woodlands out of the Forest. Despite of population increase, size of 5 main herds stayed stable during the project (51,7 in 2003-2006 and 55.9 in 2007-2010). Before the project increasing number of herd size was observed. When the two largest, intensively fed herds are analyzed, their size decreased from 77.8 in 2003-2006 to 67.5 during the project (2007-2010). When analyzed only during the project the herd size was 69 in 2006, 72.5 in 2007, 69 in 2008 and 61 in 2009. Substantial growth was observed in number of bison out of main feeding sites on the peripheries of distribution area (Fig. 1). The number of bison increased there from 75 in 2006 to 147 in 2009 – 96% growth. This increase was an effect of dispersion of bison from main feeding sites and dispersion of population in general stimulated by creation of new feeding sites and less restrictive control of bison migrating out of main forest complex. Also number of males out of feeding sites increased from 36 in 2006 to 49 in 2009 – 36% increase. In same time number of bison in main feeding aggregations declined from 291 in 2006 to 255 in 2009 – 12% decline.

Number of bison mixed herds increased from 7 in 2006 to 12 in 2009 (Fig. 2 and 3). Increase in herd number was an effect of split and dispersion of large winter herds. New herds usually stopped at meadows with hay reclaimed and mowed in frame of LIFE project. We expect that dispersion of bison, increase of herd number and decline of main herds size will

continue, as bison are quite conservative and show strong fidelity in utilization of winter feeding sites. The results of the project are even better than expected.

Table 1. Number of bison and increase rate (in comparison to 2005) of the population in Białowieża Forest in 2005-2009.

Sex/age of bison	Year				
	2005	2006	2007	2008	2009
Adult males (> 4 years old)	107	114	113	127	126
Females (> 4 years old)	152	154	158	168	181
Subadults (2-3 years old)	64	80	94	108	92
Calves	74	54	74	53	52
Total	397	402	439	456	451
Increase rate		1.2%	10.5%	14.9%	13.6%

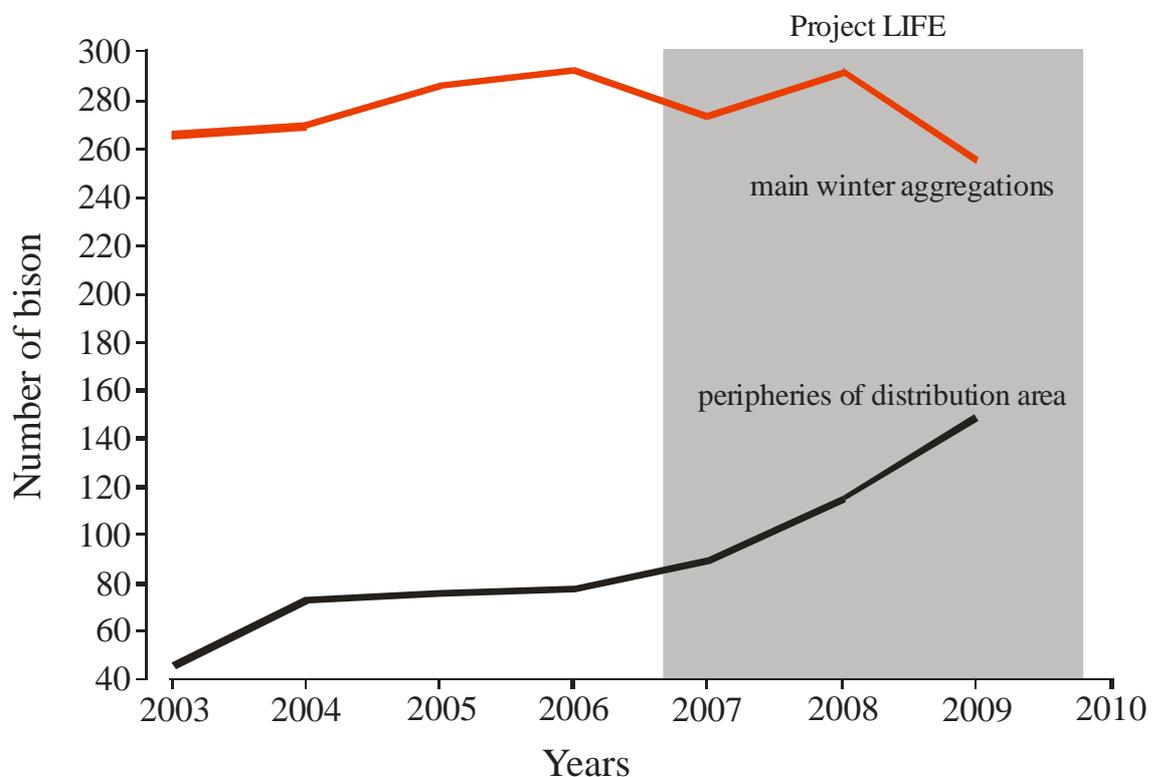


Fig. 1. Number of European bison in core area and on the peripheries of distribution area in Białowieża Forest in 2005-2009.

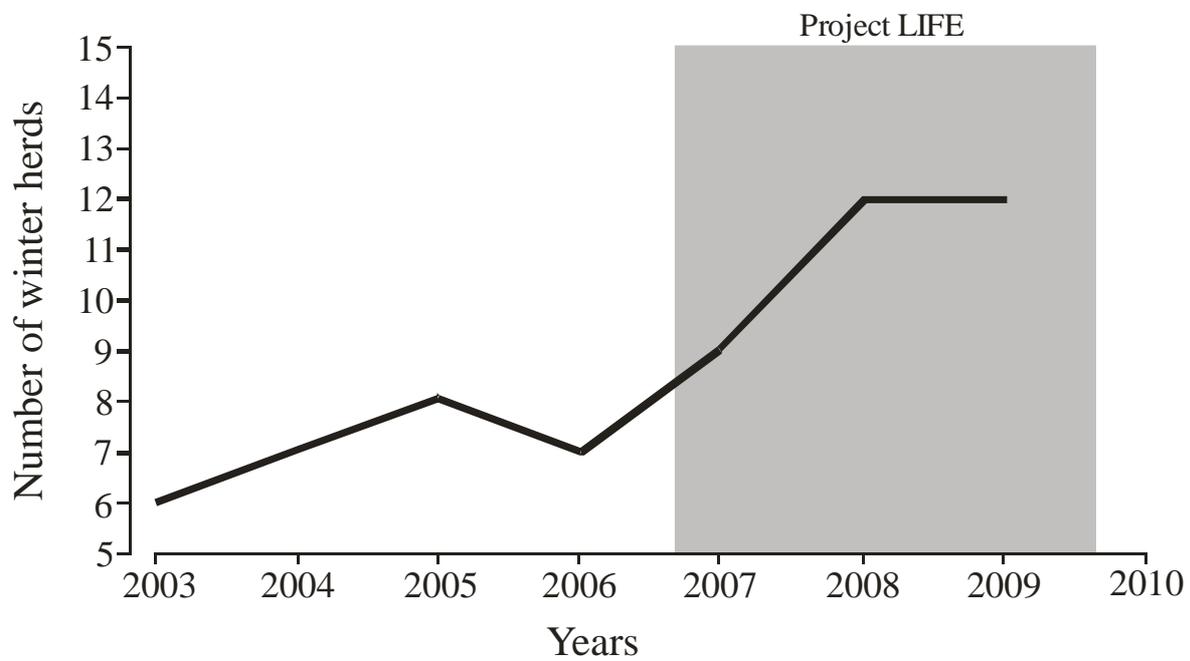


Fig. 2. Increase in number of European bison herds in Białowieża Forest

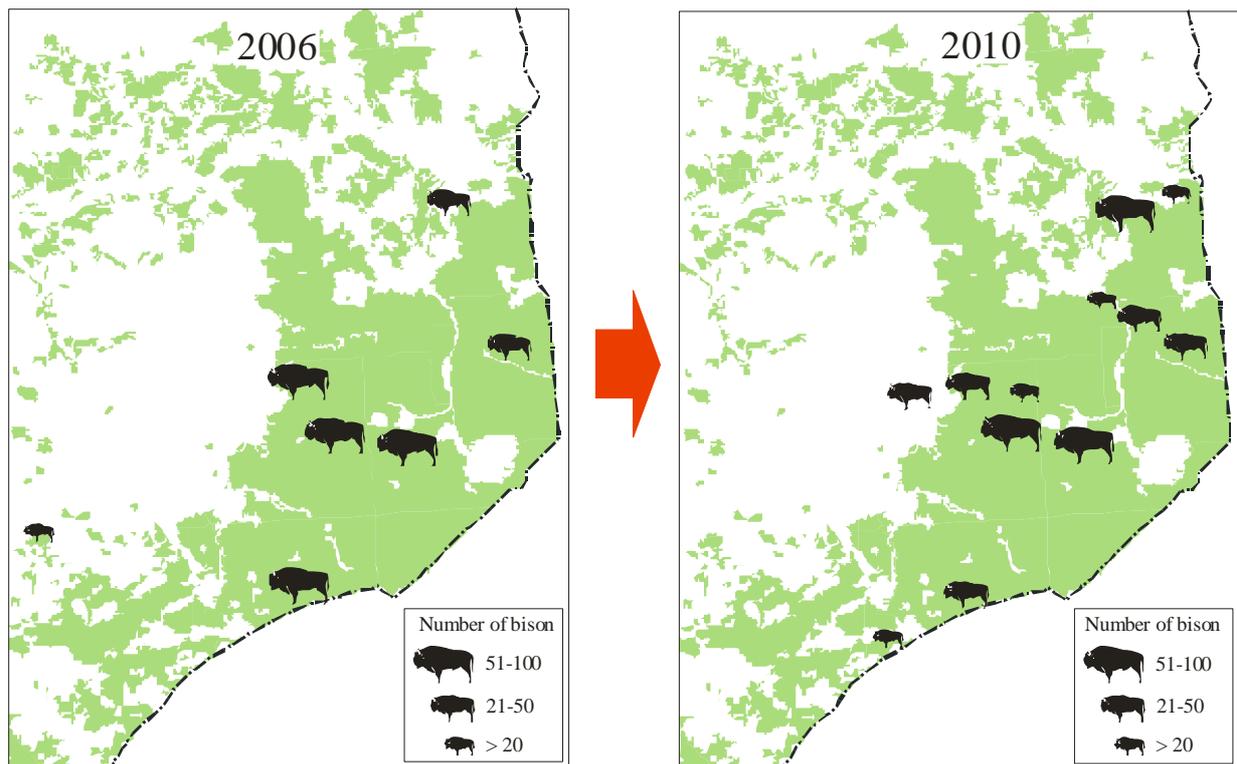


Fig. 3. Distribution of bison herds in winter in Białowieża at the beginning (2006) and at the end of the project (2009).

### Spatial monitoring

Monitoring of bison distribution and space use was conducted by radio-tracking of bison fitted with GPS and VHF collars. In total, during the project 35 bison were radio-tracked. We collected over 45 000 localizations of bison wearing GPS collars and 6 000 localization of bison wearing VHF collars.

### Space and habitat use

In parts of the Forest with high meadow density, ranges were significantly smaller (22 km<sup>2</sup>, on average) than in areas of continuous forest (68 km<sup>2</sup>). It seems that reclamation of meadows significantly improved foraging conditions. Bison in Białowieża Forest showed a very concentrated pattern of space use. Core areas with 95% and 50% probability of their occurrence calculated with Kernel method covered on average 40 and 1 km<sup>2</sup>, respectively, i.e 20 and 0.5% of their total home range used by bison (Fig. 4). Core areas overlapped with areas of meadows, which were strongly selected by bison (Fig. 5). Coniferous and mixed

forests were avoided, fresh and wet deciduous forests and other open areas were utilized respectively to their occurrence in bison ranges. Strong preference for meadows probably results from higher productivity (biomass of vegetation) than in other habitats. Meadows were especially selected in late summer and autumn (August-October), when vegetation in forest habitats is decaying. The results of this and previous studies indicate that the most suitable bison habitats are mosaics of deciduous forests and meadows; bison need to be regarded as species of mixed habitats. This approach opens new possibilities for conservation management and the reintroduction of bison in habitats other than closed forest.



Fig. 4. Space use by radio-tracked bison in Białowieża Forest



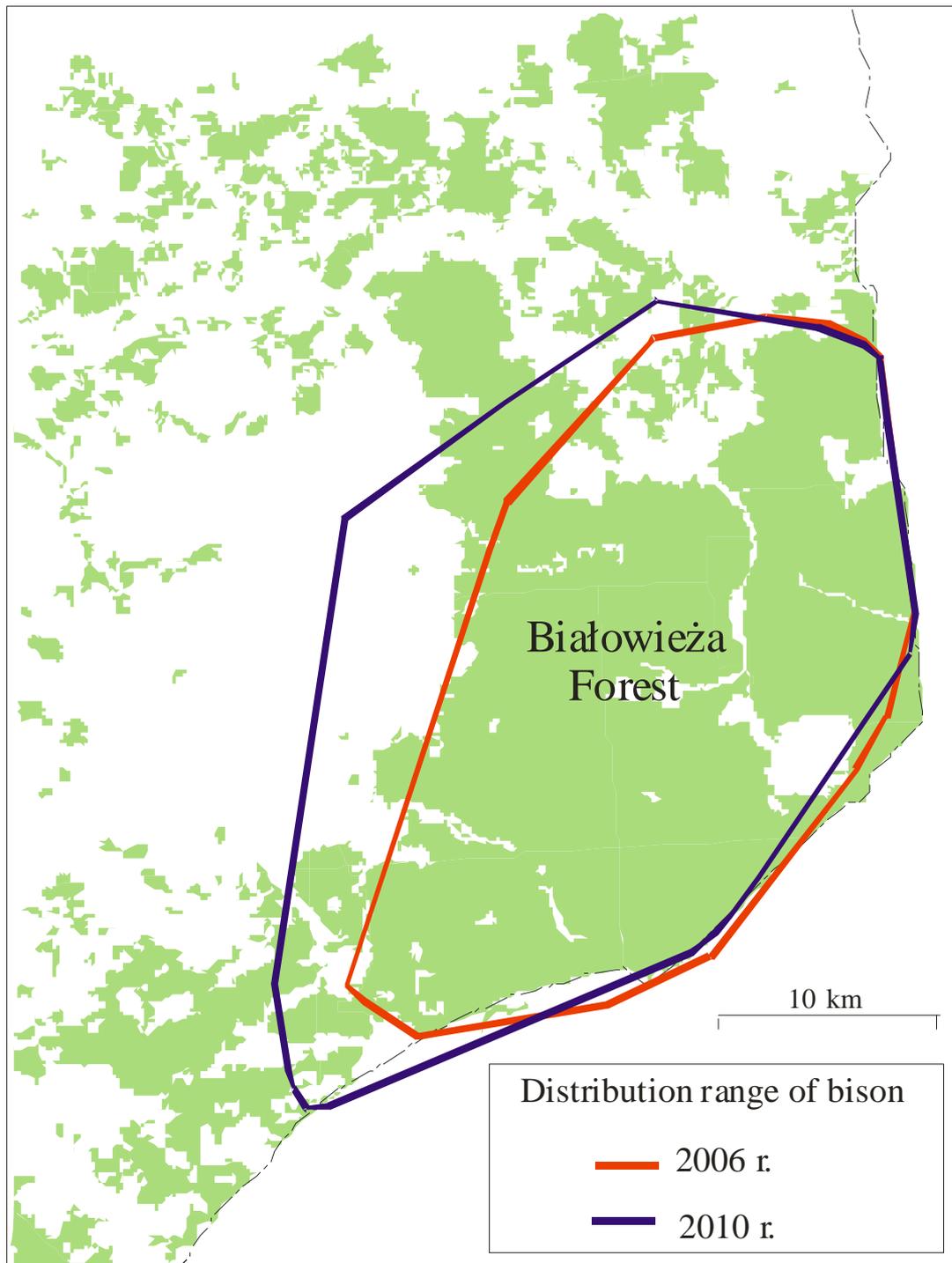


Fig. 5. Extension of bison distribution range in Białowieża Forest in years 2006-2009.

## GENETIC ASSESSMENT

The European bison (*Bison bonasus*, L.) is a symbol of successful re-introduction of a severely bottlenecked species. During the species restitution in the 1920s, only seven

individuals founded the contemporary Lowland (Białowieża) line of the European bison, and nearly 90% of the genes in the contemporary population derive from only two founders.

The studies of genetic diversity of the species conducted in 2007 and supported by LIFE financial instrument of the European Community, were focused on two sources of genetic information: autosomal microsatellite loci and mitochondrial DNA. The attempts resulted in two manuscripts being currently reviewed.

### **Microsatellite data**

Temporal variability of 21 microsatellite markers was analyzed for 178 individuals born between 1955 and 2005. The samples were divided into 5 temporal samples depending on the birth year. Genetic parameters were estimated for each temporal sample and each locus, as well as overall means for the whole population. No significant temporal differences were observed after species re-introduction for most of the genetic parameters except for the inbreeding coefficient ( $F_{IS}$ ). Mean estimation of the inbreeding coefficient  $F_{IS} = -0.02$  indicated no actual inbred mating in the population during the last 50 years. Low mean allele number per locus (2.1) and low mean heterozygosity (0.23) were observed. Changes of allelic frequencies in consecutive temporal samples resulted in a low, but highly significant overall  $F_{ST}$  value. Noticeable temporal changes of genetic diversity ( $H_E$ ) due to allelic frequency differences were detected at seven microsatellite loci, five of which decreased in heterozygosity. Although the genetic diversity of European bison after the bottleneck and re-introduction is very low, which is in concordance with expectations, we found no significant differences among temporal samples, indicating lack of unambiguous signal of further, post-bottleneck loss of genetic diversity by the species.

The detection of a post-bottleneck decrease in the frequency of rare alleles frequency in the Białowieża Forest population of European bison should spur its conservationists to minimize the further loss of genetic diversity. The most needed action for the free-roaming population in Białowieża Forest is to let it grow in number as well as let it, if possible, migrate into adjoining forest complexes. This process has been supported in Polish part of Białowieża Forest for a few years by The European Bison Programme, financed by EU initiated by Mammal Research Institute, Polish Academy of Sciences and carried out by local governmental and scientific institutions. European bison individuals from the Polish part of Białowieża Forest already form minor herds in neighbouring forested areas (Puszcza Knyszyńska, Puszcza Mielnicka and Puszcza Borecka). The border fence between the Polish and Belarussian parts of the Białowieża Forest was built in the 1980s by the Soviet Union and

since that the both Białowieża Forest bison populations have not seemed to meet. The idea of pulling down the border fence have seemed, until recently, the easiest and most effective way for actual support the genetic potential of the species, since there are nearly 300 E. bison ranging on the Belorussian part of the Forest. These animals, progeny of Polish European bison from Białowieża, were re-introduced to Belorussia in 1950s. But recent genetic analyzes of the European bison from Belorussian part of Białowieża Forest, based on microsatellite markers analyzes, revealed substantial quality differences from Polish population in microsatellite alleles composition. The effective population size ( $N_e$ ) of Belorussian population of European bison is considerably bigger than on Polish part of the Białowieża Forest ( $N_e = 32$  and  $N_e = 22$ , respectively), and the alleles found in the Belorussian European bison ( $N = 24$ ) and not present in Polish population ( $N = 275$ ) are also present in Lowland – Caucasian line, suggesting hybrid origin of the Belorussian European bison population (Tokarska et al., unpublished). In the light of these results, Polish European bison from Białowieża Forest remain the only “demographically safe” population of pure Lowland line European bison. The idea of removing the border fence and merging both populations from Białowieża Forest is then very problematic, especially in the context of the purity of the Lowland line of European bison and the presence of inbreeding depression in the Lowland – Caucasian line.

### **Mitochondrial DNA data**

We analyzed a 1429-bp fragment of mitochondrial (mt) DNA (including the D-loop region) in 118 individuals of European bison from a free-living population in Białowieża Primeval Forest. The intraspecific comparison of European bison mtDNA sequences showed very small differences. Only three distinct haplotypes differing in length within the homopolymeric cytosine tract (polyC) were observed. Heteroplasmy involving these three haplotypes was observed in 31 European bison. Furthermore, heteroplasmy concerning single nucleotide substitutions was identified in 63 samples at a total of 76 nucleotide positions. We performed cloning of three mtDNA samples of European bison in order to confirm of expected heteroplasmy. Thirty four variable sites were revealed, including six insertion/deletions of a single base pair as well as 28 single nucleotide substitutions. We also compared mtDNA sequences of European and American bison using the reference sequence of domestic cattle. An assessment of the distribution of variable positions indicated presence of two variable regions. One of these regions occur at the beginning of the D-loop region

containing over 67% of the 169 polymorphic sites and the second situated at the end of the D-loop at positions from 106 bp to 350 bp. Phylogenetic tree reconstructions in our study showed that sequences of mtDNA haplotypes of European bison were more closely related to domestic cattle *Bos taurus* than to American bison sequences despite the fact that *Bos* and *Bison* species separated about 1 million years ago based on sequence divergence. The low mtDNA variability in European bison is in concordance with theoretical expectations for this species, which has undergone an extreme and recent bottleneck followed by a reintroduction.

In spite of the fact that some important data about the genetic constitution of the European bison are already gathered, there is a serious need to continue studies including analysis of neutral markers as well as functional genes. A necessary action is temporal monitoring of the genetic diversity of the Lowland line. Temporal genetic screening is a powerful tool for monitoring different aspects of a population like individual identification, genetic structure and variation, pedigree control, migrations, presence of pathogens and diseases, breeding systems.. Low genetic diversity ( $H_E = 0.23$  and  $A = 2.1$ ) of E. bison population substantially hinders the attempts of individual identification or pedigree control of wild population, yet temporal genetic monitoring allows temporal estimations of genetic parameters of the species and is recommended for mating system planning in the European bison captive breeding centres and for wild animals management.

### **SNP data**

SNPs hold the potential to significantly expand our ability to survey both neutral (non-coding region) variation as well as genes under selection (coding region) in natural populations, providing also broader genome coverage as compared to mitochondrial DNA or microsatellites. European bison has the advantage of being closely related to domestic cattle, for which a powerful SNP analysis system (BovineSNP50 BeadChip (Illumina®)) has already been developed. Thousands of genes with known function and sometimes known genome-wide localization can be simultaneously studied in many individuals. This opens new prospects for distinguish factors that affect individual genes, alleles, or nucleotides (such as, for example, natural selection) from factors affecting the entire genome (e.g., demography).

Of the 52,978 cattle SNP loci in the BovineSNP50 BeadChip (Illumina®) that amplified in EB, a total of 929 SNPs were found to be polymorphic in EB. More polymorphic SNPs were found in the wood bison (WB) and plain bison (PB) (1,524 and 1,403 SNPs, respectively). Expected heterozygosity was lowest in EB ( $H_E = 0.135$ ) followed by WB ( $H_E =$

0.197) and PB ( $H_E = 6 \cdot 0.199$ ). The low variability detected in this study agrees with theoretical expectation for populations which have undergone a severe bottleneck.

The low  $N_e$  found for European bison was an expected result as despite the rapid population growth which followed the bottleneck of European bison in the last century, the  $N_e$  of the species has only slowly increased. This is because the long term  $N_e$  is a function of the harmonic mean which is strongly influenced by the minimum population size reached.

Genetic variability of the European bison is so diminished that classical molecular methods of identity analyses (microsatellite markers analysis) entirely inapplicable in this species. We suggested the production of a panel of selected SNP markers which could be typed using e.g. Veracode (Illumina) or SNPlex (ABI) technologies to provide an efficient and reliable method for parentage and identity analysis in European bison. A panel of 960 polymorphic SNP loci provided extremely high power to determine paternity and identity in European bison and a panel of 50-60 SNP-based genetic markers that are highly informative in European bison would be sufficient to determine the relationships between individuals. The use of such subset of markers will considerably reduce the costs associated with a genetic investigation.

Bead Chip technologies were also used to reconstruct the evolutionary relationships of the different bison subspecies (European bison, Wood bison and Plains bison) and cattle.

Previous analyses conducted on mtDNA and nuclear DNA from different bovine species distinguished two separate lineages: European bison, cattle and zebu belong to one lineage, and Bison bison, yak and gaur form the second lineage. Thus, according to these findings, the genus *Bison* is polyphyletic in contrast to Y-chromosomal sequences that assign European and American bison to one clade.

Genetic distances estimated between European bison, Plains bison and Wood bison provided a relative estimate of the time elapsed since divergence in isolation. However, as a consequence of the severe bottlenecks that affected the bison, the relation between genetic distances and divergence time is expected to be confounded by the consequences of bottleneck and genetic drift. The consequences of the bottleneck in shaping the distributions of polymorphic SNPs in the bison populations might also explain why the two trees show slightly different clusters, depending on whether the divergence rates among lineages are assumed to be constant (UPGMA method) or not (NJ method). However, both trees support the Y-chromosome phylogeny and suggest that the mtDNA phylogeny could have been produced by incomplete lineage sorting or past hybridization.

## **Main achievements and future directions**

Extensive analyses of the complete, 1429 bp sequence of the control region (D-loop) performed on nearly 200 individuals showed that there are just three haplotypes, corresponding to the three female founders (Wójcik et al. 2009). The fact that the three haplotypes are still present in the Lowland line may be explained by the relatively long generation time, but may not be sustainable without intervention. Thus targeted management of the European bison herds carrying the rare haplotypes should be undertaken to reduce the high probability of one or more of these haplotypes being lost from the Lowland line.

Application of SNP chip technology allows an accurate reconstruction of the pedigree of the European bison and could eventually also be used for estimating the true relatedness between individuals kept in captivity and recorded in the pedigree book. The traditional method for making selection decisions to minimize inbreeding utilizes only pedigree information, which describes the expected relationship among individuals. With the same pedigree, however, individuals still vary greatly in the realized genetic relationship among them. Therefore, information obtained from SNP markers can be quite useful as they will provide the realized genetic relationship and can therefore be utilised to increase  $N_e$ , in a superior way than with the traditional pedigree-based methodology.

Information on the pedigree and phenotypic data on quantitative traits, which are particularly easy to get in captive populations will allow the utilization of Animal model technique which is currently the standard method for the genetic improvement of livestock. The use of animal model can make it possible to determine whether captive populations still show significant additive genetic variance for traits of ecological relevance, such as birth mass, litter size or age at first reproduction.

SNP marker information allows an innovative breeding strategy which uses marker information to select the offspring that have the greatest potential to increase both  $N_e$  and genetic variability. This approach seems to be very promising as it consists of two strategies: a) equalization of family size and b) use of marker information to select (marker assisted selection (MAS) from within a family, the offspring that have the minimum average probability of identity by descent (PIBD). This approach would be beneficial for the bison populations in which a rapid rise in  $N_e$  and avoidance of inbreeding is very desirable

In Białowieża Forest there is, on one hand, a strong need for removal of the border fence to join separated populations on both sides of the border between Poland and Belarus, but on the other hand, the danger that the only demographically safe population of Lowland

European bison may be genetically compromised. The future of the species depends on our decisions, and we should be as sure as we can be that we undertake the most appropriate ones.