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Institute of Genetics and Animal Biotechnology
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# The study of genetic diversity of brown hare population (*Lepus europaeus* pallas, 1778) in Poland using microsatellite genotyping

**Magdalena Solka[1]*, Magdalena Misiorowska[2], Nina Strzałkowska[1], Żaneta Zdanowska-Sąsiadek[1], Alireza Seidavi[3], Paweł Urbański[1]**

1 Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, Jastrzębiec, Postępu 36A, Poland

[2] Department of Promotion and Social Communication, Regional Directorate of State Forests, 38-400 Krosno, Poland

[3] Department of Animal Science, Rasht Branch, Islamic Azad University, Rasht, Iran, 413353516

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**The brown hare is one of the most popular representatives game species in Poland. These animals provide an important part in the food chain for many species of predators. Unfortunately, the brown hare populations have decreased drastically in Poland due to many threats. These animals are currently threatened with extinction, so a protection plans are necessary. Previous analyses of brown hares in Poland based only on mtDNA sequence variability. The aim of this study was to detect genetic diversity and population structure of brown hares in Poland using microsatellites. A total of 140 brown hares were collected from seven different regions in south-eastern Poland and 12 microsatellite loci was analyzed. A total number of 140 alleles were identified. The number of alleles per locus ranged from 3 (Sol33Le) to 23 (Sol30Le). From the pool of all identified alleles, 41 (29.2%) were unique for given regions. The average expected and observed heterozygosity ranged from 0.29 to 0.91 and 0.33 to 1.00, respectively. The highest genetic distance was found between the regions: Lublin and Kielce, while the lowest was detected between Białobrzegi and Kazimierza Wielka. The Structure program and unrooted phylogenetic tree analysis showed that the seven population of** hare **is largely divided into three to two different clades. The results reveal unique**

*Corresponding author: m.solka@igbzpan.pl

**genetic variation in hare populations in south-eastern Poland and provide critical information for the conservation of this species. Our research point to consider the conservation of the brown hare population in Poland and habitat restoration and a net of ecological corridors, which could help the survival and expansion of threatened hare population in Poland.**

**KEY WORDS: genetic analysis / microsatellite DNA / hunting regions / *Lepus europaeus* / hare**

The European brown hare (*Lepus europaeus* Pallas, 1778) is one of the most important game species occurring all over Europe, with the exception of northern Russia, the northern part of the Scandinavian Peninsula, most of the Iberian Peninsula and most of the Mediterranean islands [Okarma and Tomek 2008].

Unfortunately, most of the hare populations are currently threatened with extinction, generally due to advanced agriculture, disease and attacks from predators. Because the hare populations have decreased drastically in Poland, a protection plan has been developed, which includes the creation of breeding program using local hares and, thus, for conservation of this species in Poland. Many European countries have launched activities to rescue and rebuild the decreasing hare population. Among the factors impacting population size of this species are the intensified use of pesticides in agriculture, changes in crop cultivation, environmental changes causing a reduction in plant biodiversity, an increasing number of predators, disease and climate change, low or absent of genetic flow between populations, habitat loss, infectious diseases, poaching and predation by foxes and feral dogs [Van Wieren *et al.* 2006, Roedenbeck and Voser 2008]. In the recent year, the species of *Lepus europaeus* has been included in Annex III of the Bern Convention (Convention on the conservation of European wildlife and natural habitats, CETS No. 104). In the United Kingdom hares have been classified as a species of priority protection, for which a 'Species action plan' was devised (Biodiversity Action Plan) [Vaughan *et al.* 2003]. The decline in numbers of hares in Germany, Austria, and Switzerland led to an inclusion of this species in the respective national Red Lists as "close to endangered" or "at risk" [Roedenbeck and Voser 2008]. Mamuris *et al.* 2001 studied genetic diversity within the hare population in Greece. They determined the effect of breeding hares genotype on the wild populations of hares based on an analysis of mtDNA. The study of genetic diversity of hare include not only Europe. Phylogenetic analyzes of hares in China, Russia, North America and Africa, were made [Chunhua *et al.* 2005). Also Kim *et al.* 2012 determined genetic diversity and population structure of hares in Korea using polymorphic microsatellite sequences. They recommended set of markers characteristic for this species used for further study of the genetic diversity. In Poland, knowledge of the hare population structure needs updating, especially in case of a drastic decline in its numbers. In the 1960s and 1970s, Poland's annual obtaining of hare ranged from 300,000 to 700,000 individuals [Kamieniarz and Panek 2008]. For comparison, a total of 17.3 thousand hares were obtaining in the 2010/2011 season [Budny *et al.* 2011]. The rate of decreasing was the highest at the end of 1990s and the beginning of 2000s [Strzała 2008].

National data regarding hare population size comes mainly from the Polish Hunting Association. From these data indicates, that in a large proportion of hunting districts, the hare's number is low.

Previous genetic studies on hares in Poland focused on the frequency of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) as an indicator of genotoxic effects of the environment [Jaszczak *et al.* 2004]. Next studies concerned on the genetic characteristics of the species *Lepus europaeus* based on mtDNA [Strzała 2008].

Development of molecular biology techniques gave new opportunities for research, especially concerning genetic diversity. Microsatellite sequences are widely used as a genetic markers to evaluate genetic diversity in animal population [Kawka *et al.* 2010, 2012ab, Parada *et al.* 2012]. An active protection of hares and a sustainable hunting management of this species should be determined by knowledge of the population structure [Strauss and Pohlmeyer 2001].

In Poland, among the conducted activities aimed at protecting and improving the living conditions of hares are reduce predators and hunting's prohibition in areas where is about 5 hares per 1km² [Misiorowska 2010]. Also some activities of reintroduction of hares from different types of captive breeding and accommodated them in natural environment are taken. Genetic diversity and population structure have a significant impact on survivability of every species, while a reduction in number of population size will lead to genetic exhaustion as a result of genetic drift (random loss of alleles). Genetic drift and inbreeding function stronger in small populations, therefore the risk of extinction of the population increases. Reducing these processes is possible by the existence of migration between populations.

Awareness of the value of genetic resources has encouraged studies of the genetic diversity present in different game populations. Game population genetic structure has important ecological and evolutionary consequences, but also precise knowledge on population structure is required for maintaining its economic value, because management plans could be designed for particular population. Usually, population genetic studies focus on fragmented populations, endangered species or on species with complex social systems. Lower number of investigations deals with the analyses of genetic structure in continuously distributed species, such as brown hares.

Considering all available facts, in this study we used microsatellite marker analysis to reveal genetic diversity of previously unsampled population and define population genetic structure of brown hare from different regions in south-eastern Poland. These information will provide useful data for future genetic conservation and hare management plans of their restitutions and reintroductions and relevant for sustainability of brown hare populations in the Poland.

## Material and methods

### Animals

The research material was biological samples from 140 hares (20 individuals of each region). The samples were collected between 2010 and 2011, during the hunting

seasons. The following 6 regions, subsequently termed “groups”, were included in genetic analyses: Białobrzegi, Kazimierza Wielka, Kielce, Lublin, Płock, Radom and Sandomierz were studied (Fig. 1).

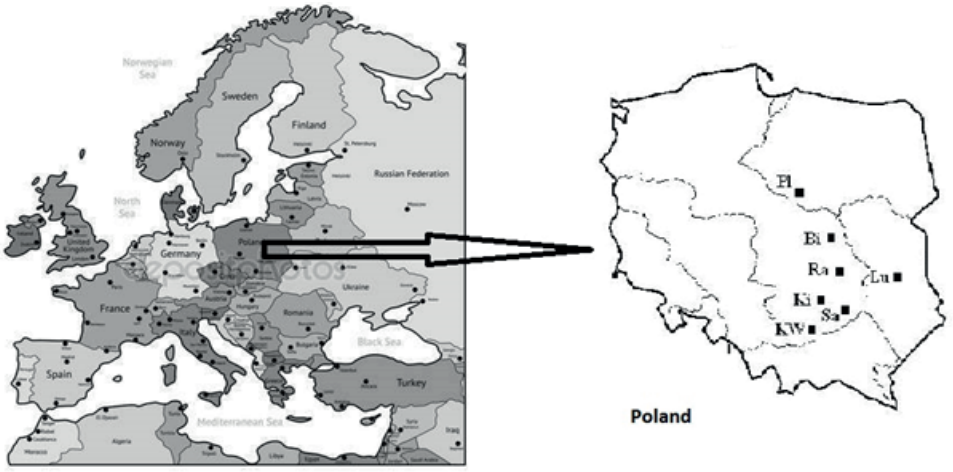



Fig. 1. Map of sampling regions. Bi – Białobrzegi, Ki – Kielce, KW – Kazimierza Wielka, Lu – Lublin, Pl – Płock, Ra – Radom, Sa – Sandomierz.

**Microsatellite analysis**

Genomic DNA was isolated from tongue tissue of hare using DNeasy Blood & Tissue KIT (QIAGEN). Each sample was examined by ND-1000 spectrophotometer (NanoDrop, USA) and electrophoresis. An analysis of 12 microsatellite sequences characteristic of hare was performed (Tab. 1).

Polymerase chain reaction (PCR) was performed in a total volume of 25 µl with 50 ng DNA templates, 50 pM each primer, 0,5 mM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl2, and 0.5 U Taq DNA polymerase (POLGEN). The PCR conditions were optimized for all 12 primer pairs. Thermal cycling began with an initial denaturation step of 94°C for 5 min, 35 cycles at 94°C for 45 s, the locus-specific annealing temperature (54-65°C), 72°C for 90 s, and concluded with final extension at 72°C for 10 min. Amplification was conducted in a thermal cycler PTC-200 Engine (MJ Research).

The lengths of amplified DNA fragments were determined using an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Forward primers were 5’- labelled with different fluorescent reporter dyes (6-FAM, VIC, NED, PET). The results were visualized and the genotyping was completed with GenScan software. The fragment sizes and alleles were determined using GeneMapper and the Genetic Analyzer software (Applied Biosystem).

**Table 1.** Characteristics of 12 hares microsatellite loci used in the study

| Microsatellite | Sequence of microsatellite | Length of alleles (bp) | GenBank no. |
|---|---|---|---|
| Sol03Le | ACCATTTGGGGAGTAGTACAGCAGA<br>CAATGGCTGTAAATGTTTCCCCTGC | 172-262 | AF191802 |
| Sol08Le | ATTGGGCCCTTTGCTCAC<br>CGCAGCCATATCTGAGAGAA | 92-122 | AF19103 |
| Sol28Le | CTGGGGAATGAACCAGTGAA<br>AATTTATTTGCAGGGCAGAGTT | 78-136 | AF538331 |
| Sol30Le | CCGAGCCCCAGATATTGTTA<br>TGCAGCACTTCATAGTCTCAGG | 148-200 | AF191804 |
| Sol33Le | TGGGCAAGGGGGAGAATCCCA<br>TCCATGTGGTTGCTGTGATGTCAA | 110-120 | AF191805 |
| Sol44Le | ATTCACCAGATGACCCCCTA<br>GGTCTGAAACACAAAGCCTCA | 92-110 | AF538332 |
| Sat12 | CTTGAGTTTTAAATTCGGGC<br>GTTTGGATGCTATCTCAGTCC | 108-138 | EU7292293 |
| Sat13 | CAGTTTTGAAGGACACCTGC<br>GCCTCTACCTTTGTGGGG | 107-121 | EU729294 |
| Lc01 | AAGGCAGGAAGCTAGTTGGA<br>CAGACATGAAGTGGCAGCAG | 140-186 | GU471251 |
| Lc03 | GTTTGGCCACTTTTTCTGGA<br>GTCAACATTGGAGGCAGGAG | 152-168 | GU471253 |
| Lc12 | CCATGAATGCACACTCCAAA<br>GTAGTGAACCCTCGGATGGA | 134-156 | GU471256 |
| Lc19 | TGACACATGAGGGGTCTTCA<br>TGAGGTTTGCTGTGGATTGA | 191-227 | GU471259 |

**Statistical analysis**

Standard genetic diversity parameters, including the number of alleles ($N_A$), the allelic and genotypic frequencies, the size (bp) and frequencies (F) of the allele by locus and population, mean number of alleles per locus, the number of observed unique alleles, the observed ($H_o$) and expected ($H_e$) heterozygosity were determined using the GENEPOP program ver. 4.0 [Rousset 2008] and GENALEX program 6.1 [Peakall and Smouse 2006]. We used GENEPOP also to test for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium. The significance levels were adjusted for multiple tests using the sequential Bonferroni correction. Markov chain parameters for exact tests were set at 10 000 dememorizations, 100 batches and 5000 interations per batch [Raymond and Rousset 1995].

Allele richness ($A_R$) per locus of hare population was calculated by FSTAT ver. 2.9.3.2 [Goudet 2001], using 1000 permuted data sets. Potential null alleles and scoring errors due to stuttering and allelic drop-out were identified by Monte Carlo simulation in MICRO-CHECKER version 2.2.3 [van Oosterhout *et al.* 2004]. Polymorphism Information Content (PIC) were estimated using CERVUS v3.0 [Kalinowski *et al.* 2007]. To evaluate the extent of differences among populations, the fixation index $F_{IS}$ was calculated as an estimator of inbreeding using FSTAT ver. 2.9.3.2 program.

Traditional tests for population differentiation were performed by calculating the index of genetic variability between population - $F_{ST}$ based on allelic identity using FSTAT v.2.9.3.2 program, with the later calculated in ARLEQUIN version 3.5. [Excoffier *et al.* 2005]. Significance was determined with 1000 permutations of samples among populations. In addition, $R_{ST}$ (genetic differentiation analogous to $F_{ST}$, unbiased with respect to differences in sample size between populations and differences in allelic size between loci) was estimated using the ARLEQUIN ver. 3.5. Using the formula of Wright 1978 has calculated the average number of migrants ($N_m$) determining gene flow between populations.

We performed Bayesian analyses in STRUCTURE version 2.3.4 [Pritchard *et al.* 2000] for the genetic structure of the hare population and for the population cluster analysis. To obtain convergence of the parameter values, we run the simulations with a burn-in period of 40,000 replicates, followed by 100,000 Monte Carlo iterations, assuming that the number of clusters K could range from 1 to 10. We define the best K value that fits the structure of the data set considering the smallest value of K (the minimum number of clusters which adequately explain the data) in the increasing likelihood, capturing the major structure data and considering the ΔK method [Evanno *et al.* 2005]. The maximum likelihood number of clusters (K) was predicted using Structure Harvester [Earl and von Holdt 2012].

The genetic relationships among samples were visualized by using principal component analysis (PCA) for 12 loci based on the covariance matrix of gene frequencies using GENALEX 6.1. The strength of the relationships among the geographical populations was estimated from the genetic distances based on the genetic distance $D_A$ by methodology of Nei 1983, using the DISPAN program [Ota 1993]. A phylogenetic tree were constructed using the unweighted pair group with the arithmetic mean (UPGMA) method and N-J (neibor-joining) by Saitou and Nei 1987 using the MEGA program, version 6.0 [Tamura *et al.* 2013]. The UPGMA and N-J tree were visualized using the TreeViewX program, version 0.5. by Roderic D.M. Page.

## Results and discussion

### Genetic diversity within and between studied group of hares

The genotypes for each of the 140 Polish brown hares were determined at the 12 MS loci and was successful for all loci. All the studied markers were polymorphic across all of the studied hare populations, and the levels of polymorphism (numbers and frequencies of alleles) varied depending on the locus. The most polymorphic were loci: *Sol30Le*, *Sol03Le* and *Lc19*, as characterized by the highest number of alleles – 23, 19 and 16, respectively.

A total of 140 alleles were detected over the 12 loci, ranging from three alleles at locus *Sol33Le* to 23 alleles at locus *Sol30Le*, with an average of 11.6 alleles per locus. The highest mean number of alleles at the locus was observed in the hunting districts: Kazimierza Wielka (6.83) and Białobrzegi (6.58), but the lowest occurred in

Radom district (6.00) (Tab. 2). This shows that the population of hares from districts of Kazimierza Wielka and Białobrzegi characterized by the highest variability, while hares from Radom district the smallest genetic variability among the analyzed hares. Additionally, the difference in genetic diversity was reflected. The allelic richness (Ar) observed at each locus ranged from 2.000 in the Płock population at *Lc03* locus to 14.735 in the Sandomierz population at locus *Sol30Le*. The mean allelic richness observed at all loci was 6.71. Private allele were observed in all the population of hare. A total 41 (29.2%) alleles were found to be private to a single hunting districts (Tab. 2). Only two microsatellite loci: *Sol33Le* and *Lc03* had no private alleles in any of hare populations. The highest number of private alleles was found in the districts of Kazimierza Wielka (8), Kielce and Lublin (7), while the lowest number of private alleles was in studied hares from Białobrzegi district (4). All 12 pairs of loci examined by the Hardy-Weinberg tests, non showed significant deviation from HWE for the studied populations after the Bonferroni correction.

**Table 2.** The number of identified alleles, the mean number of alleles and private alleles at one *locus* for individual hares and varieties

| Variety | Bi | KW | Ki | Lu | Pł | Ra | Sa | Total | Private alleles/*locus* |
|---|---|---|---|---|---|---|---|---|---|
| Number of hares | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 140 | - |
| Sol03Le | 5 | 9 | 8 | 8 | 8 | 7 | 6 | 19 | 8 |
| Sol08Le | 9 | 11 | 9 | 7 | 6 | 7 | 9 | 15 | 6 |
| Sol28Le | 9 | 7 | 4 | 5 | 7 | 6 | 7 | 14 | 5 |
| Sol30Le | 12 | 13 | 14 | 9 | 13 | 11 | 10 | 23 | 4 |
| Sol33Le | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | - |
| Sol44Le | 8 | 7 | 6 | 8 | 7 | 7 | 8 | 10 | 1 |
| Sat12Le | 7 | 7 | 6 | 7 | 7 | 6 | 10 | 11 | 3 |
| Sat13Le | 6 | 5 | 5 | 5 | 5 | 4 | 5 | 7 | 1 |
| Lc01 | 6 | 5 | 5 | 6 | 4 | 7 | 4 | 9 | 2 |
| Lc03 | 4 | 3 | 3 | 3 | 3 | 2 | 3 | 5 | - |
| Lc12 | 4 | 5 | 5 | 5 | 4 | 5 | 5 | 8 | 3 |
| Lc19 | 8 | 7 | 8 | 8 | 7 | 7 | 8 | 16 | 8 |
| Total | 81 | 82 | 76 | 74 | 74 | 72 | 78 | 140 | 41 |
| Mean number of allele at locus | 6.58 | 6.83 | 6.33 | 6.16 | 6.16 | 6.00 | 6.50 | 11.6 | - |
| SE | 0.69 | 0.86 | 0.89 | 0.57 | 0.79 | 0.67 | 0.73 | 0.67 | - |
| Private alleles/population | 4 | 8 | 7 | 7 | 5 | 5 | 5 | 41 | - |

Bi – Białobrzegi, KW – Kazimierza Wielka, Ki – Kielce, Lu – Lublin, Pł- Płock, Ra – Radom, Sa – Sandomierz.

The MICRO-CHECKER analysis, with the letter calculated using EM method [Dempster *et al.* 1977] in GENEPOP version 4.0, revealed that some loci could have been influenced by one or more null alleles. Our data demonstrated that two loci (Sol03 and Lc01) were affected by null alleles with low frequency in three studied group of hares (Kielce, Radom and Sandomierz). Our frequencies of null alleles were in the uncommon to rare range ($r<0.2$) where their effects on genotypic disequilibrium are demonstrably very low or not at all. Values of $r<0.2$ are not expected to cause significant problems in analyses [Chapuis and Estoup 2007]. All three populations

with locus Sol03 and Lc01, showing signs of a null alleles were in Hardy Weinberg equilibrium. Therefore we retained these two loci for subsequent analyses. No genotyping errors from allele dropouts or stuttering affected the allele scoring (P>0.05).

Based on the frequency of individual alleles for the 12 studied microsatellite loci was estimated the observed heterozygosity ($H_o$), which included heterozygous genotypes and the expected heterozygosity ($H_e$), taking into consideration the number and frequency of alleles and the polymorphic information content (PIC). Heterozygosity is an important parameter to evaluate genetic diversity of the population, which determines its genetic potential and adaptive capacity. The values of observed heterozygosity ($H_o$) ranged from 0.33 (Białobrzegi and Sandomierz) to 1.0 in all districts (Tab. 3). The mean value of $H_o$ for all loci was 0.85. The highest, averaged over all loci values of observed heterozygosity ($H_o$) were observed in the population of hare from districts: Płock (0.90) and Lublin (0.88), while the lowest for the hares from the Sandomierz district (0.82), which was consistent with values of polymorphic information content (PIC). The expected heterozygosity $H_e$ ranged from 0.29 for locus *Sol33Le* to 0.91 for locus *Sol30Le* (Tab. 3). The mean expected heterozygosity amounted to 0.71 per locus. The highest mean $H_e$ values were observed in the case of hares from Białobrzegi and Kielce districts (0.73), while the lowest for hares from district Sandomierz – 0.70.

Also in Table 3, the value of polymorphic information content (PIC) are presented. PIC is a parameter that measures the ability of a given marker to detect polymorphism and therefore has enormous importance in selecting markers for genetic studies. We performed analysis of PIC that checked our microsatellite loci. The

**Table 3.** The observed ($H_o$) and expected ($H_e$) heterozygosity and polymorphism information content (PIC) in the examined hares and varieties

| *Locus* | Bi | | | KW | | | Ki | | | Lu | | | Pł | | | Ra | | | Sa | | | $\hat{H}_e$ | $\hat{H}_o$ | PIC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $H_e$ | $H_o$ | PIC | $H_e$ | $H_o$ | PIC | $H_e$ | $H_o$ | PIC | $H_e$ | $H_o$ | PIC | $H_e$ | $H_o$ | PIC | $H_e$ | $H_o$ | PIC | $H_e$ | $H_o$ | PIC | | | |
| Sol03Le | 0.69 | 0.86 | 0.636 | 0.81 | 0.73 | 0.798 | 0.82 | 0.40 | 0.804 | 0.78 | 1.00 | 0.760 | 0.79 | 0.86 | 0.768 | 0.76 | 0.86 | 0.730 | 0.77 | 0.73 | 0.739 | 0.77 | 0.77 | 0.781 |
| Sol08Le | 0.85 | 1.00 | 0.839 | 0.87 | 1.00 | 0.865 | 0.85 | 1.00 | 0.834 | 0.78 | 0.93 | 0.760 | 0.78 | 1.00 | 0.754 | 0.84 | 1.00 | 0.818 | 0.86 | 1.00 | 0.844 | 0.83 | 0.99 | 0.863 |
| Sol28Le | 0.83 | 1.00 | 0.815 | 0.81 | 0.93 | 0.793 | 0.70 | 1.00 | 0.650 | 0.68 | 0.86 | 0.636 | 0.77 | 1.00 | 0.743 | 0.70 | 1.00 | 0.673 | 0.68 | 0.93 | 0.639 | 0.73 | 0.96 | 0.763 |
| Sol30Le | 0.86 | 1.00 | 0.853 | 0.85 | 1.00 | 0.846 | 0.91 | 1.00 | 0.904 | 0.85 | 0.93 | 0.833 | 0.88 | 1.00 | 0.872 | 0.84 | 1.00 | 0.833 | 0.85 | 0.93 | 0.838 | 0.86 | 0.98 | 0.906 |
| Sol33Le | 0.29 | 0.33 | 0.271 | 0.42 | 0.53 | 0.383 | 0.49 | 0.60 | 0.445 | 0.53 | 0.73 | 0.474 | 0.55 | 0.80 | 0.484 | 0.49 | 0.66 | 0.445 | 0.45 | 0.60 | 0.392 | 0.46 | 0.60 | 0.426 |
| Sol44Le | 0.84 | 1.00 | 0.819 | 0.80 | 1.00 | 0.775 | 0.72 | 1.00 | 0.683 | 0.81 | 0.93 | 0.790 | 0.79 | 1.00 | 0.765 | 0.80 | 1.00 | 0.774 | 0.83 | 1.00 | 0.809 | 0.79 | 0.99 | 0.813 |
| Sat12Le | 0.83 | 1.00 | 0.812 | 0.81 | 1.00 | 0.792 | 0.78 | 1.00 | 0.757 | 0.80 | 1.00 | 0.777 | 0.80 | 1.00 | 0.781 | 0.77 | 1.00 | 0.737 | 0.84 | 1.00 | 0.821 | 0.80 | 1.00 | 0.815 |
| Sat13Le | 0.77 | 0.93 | 0.739 | 0.66 | 0.86 | 0.621 | 0.70 | 1.00 | 0.664 | 0.66 | 0.86 | 0.614 | 0.56 | 0.66 | 0.527 | 0.56 | 0.66 | 0.486 | 0.52 | 0.60 | 0.491 | 0.63 | 0.79 | 0.636 |
| Lc01 | 0.72 | 0.66 | 0.682 | 0.51 | 0.40 | 0.483 | 0.72 | 0.86 | 0.673 | 0.76 | 0.66 | 0.729 | 0.67 | 0.60 | 0.607 | 0.78 | 0.53 | 0.751 | 0.57 | 0.33 | 0.519 | 0.67 | 0.57 | 0.691 |
| LC03 | 0.60 | 1.00 | 0.528 | 0.61 | 1.00 | 0.535 | 0.55 | 1.00 | 0.460 | 0.52 | 0.93 | 0.418 | 0.53 | 1.00 | 0.421 | 0.49 | 0.93 | 0.373 | 0.53 | 1.00 | 0.421 | 0.54 | 0.98 | 0.463 |
| Lc12 | 0.64 | 0.86 | 0.590 | 0.59 | 0.80 | 0.540 | 0.66 | 0.53 | 0.604 | 0.70 | 1.00 | 0.657 | 0.71 | 1.00 | 0.663 | 0.70 | 1.00 | 0.666 | 0.67 | 1.00 | 0.614 | 0.66 | 0.88 | 0.646 |
| Lc19 | 0.80 | 0.66 | 0.771 | 0.79 | 0.80 | 0.763 | 0.81 | 1.93 | 0.795 | 0.81 | 0.73 | 0.790 | 0.81 | 0.93 | 0.792 | 0.80 | 0.73 | 0.772 | 0.83 | 0.80 | 0.814 | 0.80 | 0.79 | 0.825 |
| $\hat{H}$ for variety | 0.73 | 0.86 | 0.696 | 0.71 | 0.83 | 0.683 | 0.73 | 0.86 | 0.689 | 0.72 | 0.88 | 0.687 | 0.72 | 0.90 | 0.681 | 0.71 | 0.86 | 0.671 | 0.70 | 0.82 | 0.662 | 0.71 | 0.85 | 0.719 |

Bi – Białobrzegi, KW – Kazimierza Wielka, Ki – Kielce, Lu – Lublin, Pł – Płock, Ra – Radom, Sa – Sandomierz.

lowest values of PIC (0.27) were recorded for locus *Sol33Le* (Białobrzegi), while the highest value of this parameter was observed for locus *Sol30Le* (0.90 – Kielce) (Tab. 3).

For a more comprehensive interpretation of the heterozygosity level and to eliminate Wahlund's effect (reduction of heterozygosity in the population due to a combination in the sample of studied subpopulations with different allele frequencies) the inbreeding coefficient $F_{IS}$ was calculated (Tab. 4). $F_{IS}$ values in all studied group of hares proved to be negative and was not significantly different (P>0.05). The presented values indicate of lack of inbreeding in the studied population of hares.

In order to estimate the genetic variability between the studied population, the genetic differentiation index $F_{ST}$ was calculated. Additionally, the genetic differentiation analogous to $F_{ST}$, unbiased with respect to differences in sample size between populations and differences in allelic size between loci – $R_{ST}$ was estimated. The interpretation of results obtained for the $F_{ST}$ index were accepted after Hartl and Clark 1997. Values below 0.05 indicate a small differentiation between populations, values ranking between 0.05 and 0.15 – a medium variability, values ranking between 0.15 and 0.25 – a high variability, while values exceeding 0.25 indicate a very high differentiation between the populations compared.

Global $F_{ST}$ was low 0.028, while pairwaise values ranged from 0.019 to 0.031 (Płock/Kielce and Lublin/Kielce, respectively) - Table 5. General $R_{ST}$ was 0.032, while pairwaise values ranged from 0.000 (Płock/Kazimierza Wielka, Sandomierz/Kazimierza Wielka and Sandomierz/Płock) to 0.193 (Lublin/Białobrzegi). Only five comparisons were significant after Bonferroni corrections

**Table 4.** Values of the inbreeding coefficient $F_{IS}$

| Varieties | $F_{IS}$ |
|---|---|
| Białobrzegi | -0.17 |
| Kazimierza Wielka | -0.14 |
| Kielce | -0.16 |
| Lublin | -0.18 |
| Płock | -0.24 |
| Radom | -0.20 |
| Sandomierz | -0.14 |

P>0.05.

**Table 5.** Pairwise $F_{ST}$ estimates and $R_{ST}$ estimates (below the diagonal) for the analyzed hare populations. Numbers in parenthesis indicate $R_{ST}$

| | Bi | KW | Ki | Lu | Pł | Ra |
|---|---|---|---|---|---|---|
| KW | 0.020; (0.012) | | | | | |
| Ki | 0.021; (0.008) | 0.023; (0.013) | | | | |
| Lu | 0.027**; (0.193)* | 0.030**; (0.084)** | 0.031**; (0.152)* | | | |
| Pł | 0.025; (0.011) | 0.030; (0.000) | 0.019; (0.005) | 0.023; (0.085) | | |
| Ra | 0.023**; (0.105)* | 0.025**; (0.051)** | 0.022; (0.059) | 0.021; (0.058) | 0.022; (0.021) | |
| Sa | 0.023; (0.047) | 0.022; (0.000) | 0.022; (0.016) | 0.027; (0.059) | 0.022; (0.000) | 0.026; (0.013) |

*P<0.05; **P<0.01 after 435000 permutations and standard Bonferroni correction.
Bi – Białobrzegi, KW – Kazimierza Wielka, Ki – Kielce, Lu – Lublin, Pł – Płock, Ra – Radom, Sa – Sandomierz.

(P<0.05 and P<0.01, Tab. 5). The statistical significance of such small values is indicative of the high level of resolution achieved by our sample size and the high variability at the MS loci.

These two statistics yielded similar results, suggesting that the level of genetic heterogeneity among these hare populations was small.

Gaggiotti *et al.* 1999 indicate that overall $F_{ST}$ estimates are more reliable than $R_{ST}$ when fewer than 20 MSs are used. Therefore, based on results of genetic differentiation $F_{ST}$, the level of gene flow between populations was determined. Generally, it is believed that population is characterized by high gene flow when $N_m$>1 [Slatkin and Barton 1989]. The average number of migrants ($N_m$) was calculated by the formula Wright 1978 and for all the hare populations was 8.758. This indicates that is gene flow between our populations.

**Relationship among studied group of hares**

On the basis of the matrix of $F_{ST}$ values, principal component analysis (PCA) for 12 loci was conducted. In the obtained image of grouping hare population first component explains 26.45%, the second component of the index - 20.14% and the third – 17.31% of the observed genetic variation. The PCA shown in Figure 2 that most of studied group of hares cluster within two major groups and 46.59% of the variation is explained by just two main components PC1 and PC2. The most distant outliers in the PCA (Kazimierza Wielka, Płock and Lublin) are in the farthest locations.

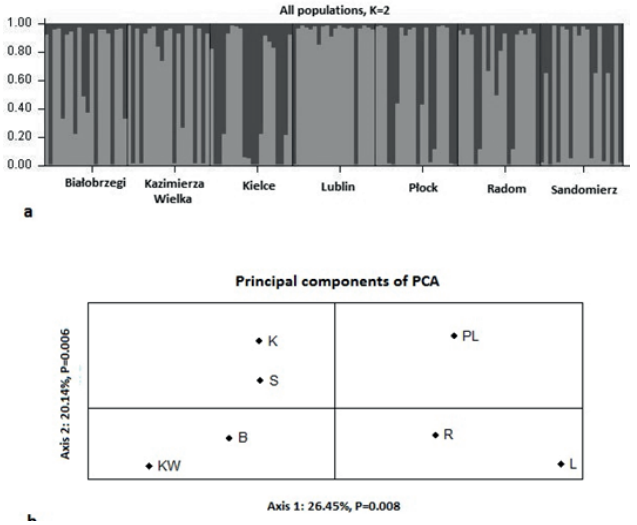
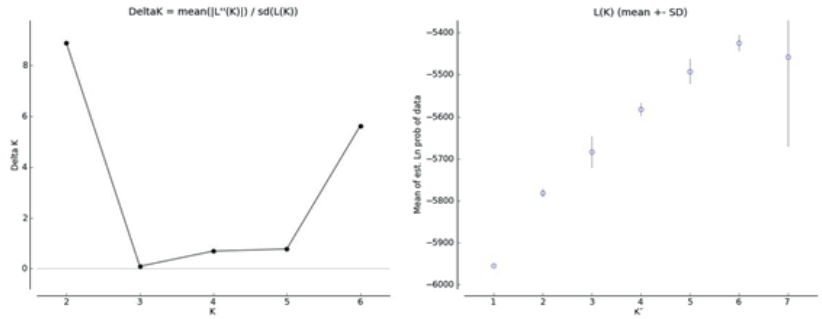



Fig. 2. a – Results of individual assignment based on Bayesian cluster analysis of the studied hare populations obtained with STRUCTURE by highest likelihood when K = 2. Individuals of different hunting districts are separated by black segments. Each individual is represented by a vertical line corresponding to its membership coefficient, that is a solid line corresponds to a membership coefficient of one (indicating no admixture). b – principal component analysis (PCA) based on pairwise FST.

**Population structure of the studied hares**

To estimate the genetic structure of the hare population, we performed population structure analysis based on 12 markers with the Bayesian method and the STRUCTURE

software. The Bayesian clustering method was used to look for genetic structure using only genotypic data, this approach considered each animal independently, regardless of the population grouping within which the hare was sampled. Hierarchical population structuring were further analyzed with STRUCTURE. We were run multiple times using the admixture model, under the hypotesis that the number of populations (K) was between 1 and 7. We used the log $P(x|K)$ to choose the minimum number of clusters which adequately explained the data. We chose a burn-in period of 40 000 replicates, followed by 200 000 iterations of the Markov chain and did five replicates of the process for each '*K*'. Following the ΔK method, our data set of seven groups of hare was best described by K=2 genetic clasters. Overall STRUCTURE results suggest that studied Polish brown hare populations compose a double genetic clusters (Fig. 3).




| K | Reps | Mean LnP(K) | Stdev LnP(K) | Ln'(K) | \|Ln''(K)\| | Delta K |
|---|---|---|---|---|---|---|
| 1 | 5 | -5955.133333 | 1.404734 | — | — | — |
| **2** | **5** | **-5781.200000** | **8.890646** | **173.933333** | **76.250333** | **8.572818** |
| 3 | 5 | -5683.560000 | 36.336587 | 97.640000 | 3.480000 | 0.095772 |
| 4 | 5 | -5582.440000 | 15.405107 | 101.120000 | 10.800000 | 0.691048 |
| 5 | 5 | -5492.120000 | 29.194609 | 90.320000 | 22.620000 | 0.774747 |
| 6 | 5 | -5424.420000 | 18.065215 | 67.700000 | 101.446667 | 5.615580 |
| 7 | 6 | -5458.166667 | 211.625298 | -33.746667 | — | — |

Fig. 3. Plot of ΔK and L(K) values computed and plotted in STRUCTURE HARVESTER.

**Genetic distance of the studied population**

In order to determine the degree of genetic relatedness of hares from different hunting districts, genetic distance $D_A$ was estimated (Tab. 6). The largest genetic distance was found between the region Kielce and Lublin, while the smallest - between Białobrzegi and Kazimierza Wielka.

The UPGMA dendrogram constructed based on the genetic distance is shown on Figure 4. An unrooted phylogenetic tree showed that all seven studied population of hare were largely divided into two clades (A and B). Although the bootstrap values for the tree are low, this UPGMA dendogram shows that, the most numerous is the clade A grouping individuals of 5 from 7 studied hare population. Populations sampled from Białobrzegi (Bi), Kazimierza Wielka (KW), Sandomierz (Sa), Kielce (Ki) and Płock (Pl) formed one cluster and differed from the two others population (Lublin-Lu and Radom-Ra). These findings were also obvious in the PCA scatter plots and STRUCTURE analysis.

**Table 6.** Genetic distance $D_A$ between the analyzed hares

| | *Bi* | *KW* | *Ki* | *Lu* | *Pł* | *Ra* |
|---|---|---|---|---|---|---|
| **KW** | 0,110 | | | | | |
| **Ki** | 0,121 | 0,133 | | | | |
| **Lu** | 0,167 | 0,185 | 0,199 | | | |
| **Pł** | 0,142 | 0,169 | 0,114 | 0,139 | | |
| **Ra** | 0,132 | 0,147 | 0,133 | 0,127 | 0,128 | |
| **Sa** | 0,126 | 0,115 | 0,122 | 0,157 | 0,126 | 0,145 |

Bi – Białobrzegi, KW – Kazimierza Wielka, Ki – Kielce, Lu – Lublin, Pł- Płock, Ra – Radom, Sa – Sandomierz.

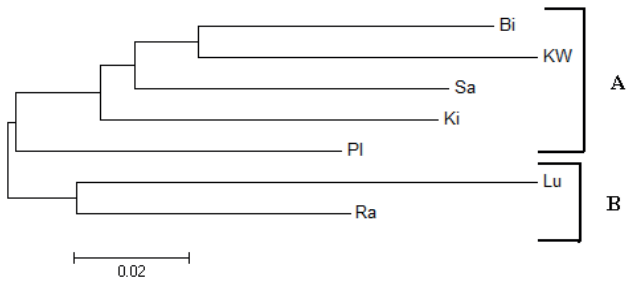



Fig. 4. UPGMA dendrogram of 7 studied populations of hare. Bi – Białobrzegi, Ki – Kielce, KW – Kazimierza Wielka, Lu – Lublin, Pl – Płock, Ra – Radom, Sa – Sandomierz, A, B - two separate clads.

All these hunting regions are grouped along the western border of southeastern of Poland. In the case of clade B can be concluded that it groups together individuals from the central part of the region of southeastern Poland, namely hares from Lublin and Radom. The phylogenetic tree based on similarities of hares obtained by microsatellite genotyping indicates that this construction not diversified precisely hares from southeastern Poland according to the geographical location of their occurrence regions. This suggests gene flow among hares in the study area.

In this study, population genetics parameters using 12 microsatellite markers were measured for hares from seven locations in the south-eastern Poland. Information provided by the microsatellite analyses in the present study clearly does reflect the genetic differentiation and distinctiveness among studied seven population of hares. The molecular analysis presented in this paper is the first attempt to compare genetic diversity (based on microsatellite DNA analysis) of seven Polish natural populations of hare.

Analysis of 12 microsatellite DNA loci confirmed the high genetic variability among the studied Polish population of hare. This is in accordance with the high level of variation identified inter alia in Ireland [Hamill *et al.* 2007], in South Africa [Suchentrunk *et al.* 2009], in Italy [Modesto *et al.* 2011] and in Greece [Antoniou *et al*. 2013].

The number of alleles at a single locus and the mean number of alleles per locus was generally high. The highest average number of alleles at the locus were observed in hares with hunting districts: Kazimierza Wielka (6.83) and Białobrzegi (6.58), while the lowest in the district of Radom (6.00). Our results were similar to those described for wild populations of *L. europaeus* – 6.6 in Andersson *et al.* 1999; from 6.5 to 8 in Estonba *et al.* 2006. Also Suchentrunk *et al.* 2009 used the thirteen microsatellite loci with different levels of polymorphism to study molecular characteristics of 66 *L. capensis*-type hares from different locations in the R.S.A. These authors revealed a total number of 137 alleles, with an average of 10.54 alleles per locus. The number of alleles per locus ranged from 3 (*Sat8*) to18 (*Sol08*), and the total number of alleles per subspecies ranged between 55 (*L. c. capensis*, *L. c. ermeloensis*) and 79 (*L. c. centralis*). They also showed that among all alleles, 30 (21.9%) were private, i.e. found in one subspecies only, whereas the others were shared between two or more subspecies. None of the thirteen loci showed a significant presence of null alleles, there was no hint of allelic drop-out, and there was no linkage disequilibrium for any pair of polymorphic loci, when tested separately for each subspecies.

In our studies the level of private alleles was similar – 41 (29.2%) constituted as private alleles. Only two microsatellite loci: *Sol33Le* and *Lc03* were not specific in any of hare populations**.** The largest number of private alleles was characteristic to a hare from districts of Kazimierza Wielka (8), Kielce and Lublin (7), while the lowest number of private alleles in studied hares occurred in the Białobrzegi district (4). Having such a large number of private alleles in a population reflects its autochthonous character. Therefore, in future constant genetic monitoring of the analyzed groups of hare based on the indicators reported in this study will be recommended. It is very important to continue genetic studies and acquire more information about the genetic condition of the analyzed populations, and construct an appropriate plan as well the strategy for conservation of hare resources in Polish forests.

In turn, Modesto *et al.* 2011 analyzed 109 samples from two brown hare populations captured in two protected areas in northern Italy. All eight microsatellite markers were observed to be polymorphic and a total of 57 alleles were found. The number of alleles per locus ranged from 2 (*Lsa6*) to 11 (*Lsa2*) – mean, 6.5.

The heterozygosity values, presented for all analyzed hares must be considered as high. The observed heterozygosity ($H_o$) value ranged from 0.33 to 1.00 whereas the expected heterozygosity ($H_e$) from 0.29 to 0.91. Similar values of heterozygosity for two hare population from northern Italy were presented by Modesto *et al.* 2011. In these studies, the observed heterozygosity ($H_o$) value ranged from 0.34 to 0.80, whereas the expected heterozygosity ($H_e$) from 0.28 to 0.80.

Our results allow to conclude that among all analyzed hares from hunting districts Kielce and Kazimierza Wielka characterized by the greatest genetic diversity (the highest values of heterozygosity, the largest number of alleles and most specific alleles).

Kim *et al.* [2012] developed and characterized nine microsatellite loci for Korean hare. The number of alleles across the two sampling regions ranged from three to nine with a mean of 6.1. Mean observed and expected heterozygosities and polymorphic information content were 0.540, 0.627 and 0.579, respectively. Next, Antoniou *et al.* [2013] assayed hare specimens from northeastern Greece and allelic data from 10 microsatellite loci was used. The analyzed markers were polymorphic having a mean number of alleles per locus equal to 15.8, ranging from 7 (*Lsa3*) to 28 (*Sol30*). For the total number of samples pooled in one population, the observed heterozygosity $H_o$ and the unbiased estimation of expected heterozygosity $H_e$ were 0.4872 and 0.7539, respectively.

Fixation index $F_{ST}$ for the analyzed hares ranged from 0.019 to 0.031. In all analyzed groups of hare were recorded a low differentiation between the analyzed hunting districts. In studies of Modesto *et al.* 2011 the $F_{ST}$ coefficient was 0.036, and showed a slight differentiation between the two populations. Also Hamill *et al.* 2007 examined genetic structure in the mountain hare in southern Ireland and they found that genetic differentiation ranged from low to moderate (pairwise $F_{ST}$ = 0-0.116). $F_{ST}$ was not correlated with geographic distance, suggesting possible population fragmentation. These results are consistent with natal dispersal of nonresident males into the sampling areas.

The average number of migrants ($N_m$) was 8.758. With the increase in the number of migrants decreases the value of $F_{ST}$, i.e. differentiation between populations. When the value of $N_m$ is greater than 10, populations are characterized by free movement of genes. When we have isolated populations in which no exchange of genes, $N_m = 0$ and $F_{ST} = 1$. In our case, the average genetic variation for 12 microsatellite loci among seven populations of hares was small, $F_{ST}$ was 0.028. This indicates that the level of gene flow between the populations is only slightly reduced.

The largest genetic distance was found between the districts Kielce and Lublin, while the smallest - between Białobrzegi and Kazimierza Wielka. The results of the phylogenetic analysis of the seven groups of hare showed a rather small distance between the groups, and indicated that the Lublin and Radom populations were the same clade. The small genetic distance and $F_{ST}$ values observed between the studied populations suggested that there is some gene flow between the populations. Phylogenetic analysis results suggested probable route of polish brown hare migrations - from south-eastern Poland to other regions. However to confirm those results further analysis of nuclear loci are necessary.

Such studies, based on genetic analyses, allow researchers to identify suitable hare material for the creation of new population. Knowledge about the genetic diversity of a newly created populations allows genetic monitoring during a restitution program and tracing genetic changes in natural populations.

## Conclusions

Genetic studies on Polish brown hare using MS DNA markers have not been carried out so far. So, our study is a first report enriching the knowledge of genetic and phylogenetic characteristics of hares from seven hunting regions in the south-eastern Poland based on their microsatellite polymorphism, which show that microsatellite markers may be suitable not only for the genetic monitoring of hare populations but also for revealing the population structure of brown hare in Poland. The obtained results provide a view of Polish hare population as high genetically diverse. The genetic structure of polish hare populations exhibits stabilization manifested equal distribution of the genetic variability resources among all the studied populations.

The present study reveals little difference between studied groups of hare in allelic variation, heterozygosity and inbreeding coefficients, indicating that the studied population have not lost a great degree of genetic variation.

The genetic biodiversity of those populations, identified through the present research, can serve as output indicators of the gene pool for breeding programs and the conservation of this species in Poland because baseline genetic data are crucial to guide future population specific conservation programs and research efforts on European brown hare in Poland.

Further studies must be conducted over time to provide a more comprehensive understanding of the genetics of this species.

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