

OVERVIEW OF GENETIC DIVERSITY OF BROWN HARE (*LEPUS EUROPAEUS* PALLAS) FROM BULGARIA

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Abstract

There are so far several studies on the level of genetic diversity and differentiation of the brown hare populations in Bulgaria. Since the information available is not summarized the aim of this review was to summarize previous knowledge on genetic diversity of the brown hares from Bulgaria in order to disclose new horizons and directions for future genetic studies. We did a review of 10 publications on the brown hare genetic diversity, mostly in Bulgaria but also in other adjacent regions, where the brown hare was introduced or naturally presented. The results from allozymic, RAPD and microsatellite markers show that the genetic diversity is the highest in the Anatolian populations, followed by the Greek, Bulgarian, Serbian and finally by the Austrian ones. A fragmentation on a small geographical scale can be seen, illustrated by the presence of regional rare alleles, appearing with a low frequency. Gene flow is present across long geographical distances. There was no negative impact on the genetic diversity, caused by decreasing of population size for long periods of time.

Key words: allelic richness, Bulgaria, heterozygosity, molecular markers, population genetics.

Introduction

The brown hare *Lepus europaeus* Pallas, 1758 is the most widely distributed and one of the most studied species of the genus (Wilson and Reeder 2005). Since 1960s the brown hare populations were decreasing all over the European continent (Smith et al. 2005), and the rate of decreasing was higher at the end of 1990s and the beginning of 2000s (Strzała et al. 2006). In Bulgaria these changes were truly tangible, especially after 2000. The hunting bag reached significantly low lev-

el, if compared to other European countries. For instance, in Poland in the early 1970's, harvesting reached 700,000 individuals and in 1990 – 505,000 (Hartl et al. 1992). In Bulgaria the respective quantities were 300,000 brown hares shot in 1970 and only 50,000 in 1990.

The brown hare was included in the Red list of threatened species of IUCN, with the category Least Concern – LC (Smith and Johnston 2008). This category also lists widespread and abundant taxa and those which do not qualify for the other categories. It is included also in Ap-

pendix III of the Convention of the Conservation of European Wildlife and Natural Habitats – Bern Convention (Vaughan et al. 2003, Smith et al. 2005), and is classified as a priority species of conservation concern by the UK government (Smith et al. 2005).

Since 1980s many European researchers started studies on the population genetics of brown hares in different habitats of the continent. Most studies were focused to test whether the reduction of population size could cause heterozygote deficiency, decreasing of the mean allele number and allelic richness, extinction of endemic alleles, and the presence of bottleneck effect and inbreeding. The level of genetic variability and differentiation of the Bulgarian brown hare populations were studied as well (Suchentrunk et al. 2000), but all these studies over the years are still not summarized. A summary and/or critical review could be in favour by giving direction for future genetic studies, which can reveal the level of the Bulgarian brown hare genetic diversity, and could serve as opportunity for assessment of an indirect correlation between genetic diversity and population size for different periods. This could help also the decision making related to the needs of translocation and restocking programs. According to the Bulgarian wildlife experts, one of the major reason for the hare stock decreasing is the lack of translocation and restocking for the last 30 years (common practices in the past), no matter if the hares were imported or just locally displaced, in order to improve the genetic diversity. The need to summarize of scientific results on the population genetics research is also determined by the lack of information on this topic in Bulgaria. Therefore, we tried to review the publications related to ge-

netic variability of the brown hare, not only in Bulgaria and Europe, but also in other areas, where it was introduced or occurs naturally. Our aim was to show the results (presented in comparative tables) of studies that include individuals of brown hares from Bulgaria.

Genetic Diversity and Differentiation of Brown Hares Based on Allozyme Gene Markers

The first and the most comprehensive population genetic study of Bulgarian brown hares (Suchentrunk et al. 2000) compared genetic diversity of 8 Bulgarian with 20 Austrian populations, the latter studied by Hartl et al. (1993). Kidney and liver samples were tested from 157 brown hares, collected in 8 country regions in the period 1993–1995 for determining of heavy metal content (Tatatruch et al. 1996), within the research program of Research Institute of Wildlife Ecology (Vienna, Austria). The results (Suchentrunk et al. 2000, Table 1) showed that there was a significant differentiation in allele frequencies between Bulgarian and Austrian populations in 6 loci of a total 50 studied. Only among Bulgarian hare populations, there was significant differentiation at 7 loci.

In Bulgarian populations polymorphism was found at 11 loci, which is very similar to the Austrian ones. Mean number of alleles also had similar values (1.16–1.18 for Bulgaria, 1.08–1.20 for Austria). There was no linkage disequilibrium between the locus pairs. Significant correlation was observed between the expected and observed heterozygosity ($p < 0.0001$). Only in two populations there was a difference in expected heterozygosity for all loci. The genetic differentiation (F_{st} values) among 8 Bulgarian populations was generally low (probably due to in-country hare translocations in the period 1970–1980). Absolute

Table 1. Comparison of genetic variability in Bulgarian (BG) and Austrian populations (Suchentrunk et al. 2000).

| Gen. ind. | Bre | D. Mit. | Vra | Ait | San | St.Z | Vid | Dob | BG Mean | BG Range | Austria Mean | Austria Range |
|-----------|-------|---------|-------|-------|-------|-------|-------|-------|---------|-------------|--------------|---------------|
| <i>N</i> | 22 | 20 | 25 | 20 | 19 | 15 | 16 | 20 | 8 | 157 | 20 | 469 |
| <i>Ho</i> | 0.017 | 0.036 | 0.033 | 0.025 | 0.042 | 0.026 | 0.029 | 0.031 | 0.03 | 0.017–0.042 | 0.030 | 0.020–0.039 |
| <i>He</i> | 0.026 | 0.046 | 0.038 | 0.026 | 0.045 | 0.031 | 0.035 | 0.034 | 0.035 | 0.026–0.045 | 0.027 | 0.022–0.033 |
| <i>P</i> | 10.2 | 14.3 | 12.2 | 10.2 | 10.2 | 12.2 | 14.3 | 12.2 | 12 | 10.2–14.3 | 10.7 | 8.2–16.3 |
| <i>A</i> | 1.14 | 1.2 | 1.18 | 1.18 | 1.16 | 1.16 | 1.16 | 1.16 | 1.17 | 1.14–1.2 | 1.13 | 1.08–1.20 |

Note: Bre – Breznik, D. Mit – Dolna Mitropolia, Vra – Vratza, Ait – Aitos, San – Sandanski, St.Z – Stara Zagora, Vid – Vidin, Dob – Dobrich; *N* – number of individuals, *Ho* – observed heterozygosity, *He* – expected heterozygosity, *P* – percent of polymorphic loci (99 % criterion), *A* – mean number of alleles per locus.

genetic differentiation occurred among regional and inter-regional samples from Bulgaria and central Europe. This implies considerable gene flow across long geographical distances leading to a rather panmictic network of local populations. Despite the general picture of a high level of gene flow over long geographic distances, there was a tendency of a slight gene pool divergence between local populations of southeastern (Bulgarian) and central (Austrian) European brown hares. Genetic studies showed also that Bulgarian hare populations are very similar, forming a relatively homogeneous gene pool, which is slightly different to other regions in Europe. A new allele was found in the population of Sandanski that was not found in Central European populations. The authors' explanation was related to the possible slight gene flow from North Greece to South Bulgaria. There were no other new alleles in Bulgarian populations, which suppose an absence of a gene flow coming from Asia Minor or North-east of the Black Sea. The results of Suchentrunk et al. (2000) concerning the distribution of allele frequencies in the populations studied reinforce the hypothesis that there was no gene flow coming

from Southeast Europe, i.e. Bulgaria, to the Central Europe.

Hare liver samples were sampled from 91 brown hare individuals in seven regions of continental Greece and Crete for the period 1998–2000. In order to compare genetic diversity of these populations with the results obtained by Suchentrunk et al. (2000) the genetic analysis were done using the same methodology and in the same laboratory (Suchentrunk et al. 2003). Three rare alleles with low frequencies are found, probably endemic, in the 35 loci studied. These alleles were not discovered in other populations in Central and South Europe (Hartl et al. 1994, Suchentrunk et al. 2000). This result indicates that during the late Pleistocene Greece was a refuge for brown hares for Southern Balkans. Three other alleles found in Bulgarian and Austrian populations were not presented in Greece (Suchentrunk et al. 2000). The alleles Pep-294, found in Bulgarian populations from the area of Sandanski by Suchentrunk et al. (2000) was not found in Greek ones which refute the hypothesis that this allele comes from Greece, by following the Valley of Struma River. Overall rates of polymorphism, i.e. percent of polymorphic

loci (no criterion) did not differ significantly ($p > 0.05$, two-sided Fisher's exact test) between Greek (14.3 %) and Bulgarian (20.0 %) hares.

Mean allele number (Table 2) in Greek populations varied weakly – 1.09–1.31 (for Bulgaria 1.11–1.20). For the Greek populations average observed and expected heterozygosity ranged between 0.04–0.05 and 0.036–0.053, respectively. All values of the inbreeding coefficient (F_{is}) were not too far away from zero. The genetic diversity between Greek and Bulgarian populations was 3.3 % and between all studied populations it was 6 %. All indices of genetic diversity did not vary significantly between Bulgarian and Greek populations, except the observed heterozygosity, which was higher in Greek populations. Generally, the Greek populations had slightly higher genetic diversity in comparison with the allozymic diversity of Bulgarian and Central European (Austrian) populations (Hartl et al. 1993, 1994; Suchentrunk et al. 1999, 2000, 2001). These results also show that the low population size existing for a long pe-

riod in Greek populations (Mamuris et al. 2001, Sfougaris et al. 1999) so far did not have a negative effect on their genetic diversity. There was very low differentiation between Bulgarian and Greek brown hare populations (Suchentrunk et al. 2003). The low levels of allozymic demarcation are often normal within the populations of brown hare and other *Lepus* species, even for large geographical distances in Europe (Hartl et al. 1993, 1994; Suchentrunk et al. 1999, 2000, 2001). One of the reasons for the small difference in genetic diversity in Bulgaria and Greece can be explained by the translocation of hares in the past. Indeed, in a preserved reports of Greek ministry of Agriculture there is information that during 1990's there was a big amount of brown hares imported from Bulgaria, raised in Greek farms and then released (Mamuris et al. 2002). The observed low level of nuclear gene pool differentiation in Greek and Central European populations confirms the above mentioned conclusion.

Genetic diversity of 57 brown hares from Anatolia (Sert et al. 2005) was compared with 717 brown hares from

Central Europe – Austria (Suchentrunk et al. 2000), Southeastern Europe – Greece (Suchentrunk et al. 2003) and Bulgaria (Suchentrunk et al. 2000), in another study performed in the period of 2001–2003. The common 28 loci for all populations were compared of 48 in total (Table 3). The results showed, that of 48 loci assayed, 19 (39.6 %) were polymorphic with two to four alleles in the Anatolian hares. An interesting fact

Table 2. Indices of genetic variability in brown hare populations from Greece and Bulgaria based on isozyme gene markers (Suchentrunk et al. 2003).

| Genetic indices | Bulgaria Mean | Bulgaria Range | Greece Mean | Greece Range |
|----------------------|---------------|----------------|-------------|--------------|
| <i>N</i> | 8 | 157 | 7 | 91 |
| <i>H_o</i> | 0.033 | 0.015–0.043* | 0.044 | 0.040–0.050 |
| <i>H_e</i> | 0.038 | 0.026–0.052 | 0.043 | 0.036–0.053 |
| <i>P</i> | 12 | 5.71–11.43 | 9.39 | 8.57–14.29 |
| <i>A</i> | 1.17 | 1.11–1.20 | 1.18 | 1.09–1.31 |

Note: *N* – number of individuals and populations, *H_o* – observed heterozygosity, *H_e* – expected heterozygosity, *P* – percent of polymorphic loci (95 % criterion), *A* – mean number of alleles per locus. * In the original paper (Suchentrunk et al. 2003) the values are given in percentages.

is that 14 alleles, although with a low frequencies in 13 loci were not found in European populations. Probably they could have occurred through a gene flow coming from different territories around Anatolia.

None of the four Turkish populations showed evidence for a bottleneck effect during the last generations, but there are doubts about its presence in the past due to low allele frequencies, found only in Anatolia. The comparison of population genetic indices for genetic diversity among Anatolia, Greece, Bulgaria and Austria revealed that the highest values were found in the Anatolian populations followed by Greek and Bulgarian ones, and the last were the Austrian populations. The same trend could be observed in allele frequencies and polymorphism distribution for all 28 loci. Genetic diversity among those 4 populations was 60.28 %, the other 39.72 % due to the diversity within populations. No genetic signal of inbreeding was detected in Anatolian brown hare populations contrary to the Greek and Bulgarian ones, where *F_{is}* values reached values as high as 0.373 and 0.426, respectively. Greek hares are most similar to Anatolian ones, followed by Bulgarian and Austrian hares. The genetic diversity of Anatolian populations was higher, as compared to European ones. According to Suchentrunk et al. (2003) the reason can be found in the continuous hare presence during the periods of glaciations in Greece and Ana-

Table 3. Indices of genetic variability in brown hare populations from Anatolia (Turkey), Austria, Bulgaria and Greece (Sert et al. 2005) isozyms.

| Genetic indices | Anatolia (Turkey) range | Austria range | Bulgaria range | Greece range |
|----------------------|-------------------------|---------------|----------------|--------------|
| <i>N</i> | 4 | 20 | 8 | 7 |
| <i>H_o</i> | 0.049–0.087 | 0.018–0.055 | 0.019–0.054 | 0.050–0.063 |
| <i>H_e</i> | 0.046–0.071 | 0.024–0.042 | 0.032–0.065 | 0.045–0.067 |
| <i>P</i> | 14.25–25 | 7.14–25 | 10.71–21.43 | 10.71–17.86 |
| <i>A</i> | 1.18–1.36 | 1.07–1.39 | 1.14–1.25 | 1.11–1.39 |

Note: *N* – number of populations, *H_o* – observed heterozygosity, *H_e* – expected heterozygosity, *P* – percent of polymorphic loci (99 % criterion), *A* – mean number of alleles per locus.

tolia and probably some other regions of the Balkans during Pleistocene, when the unfavorable climate conditions did not provide habitats good enough in most of the northern territories (Corbet 1986). An additional explanation comes out of the concept that Anatolia is a biogeographical crossroad, with small ecological barriers for the gene flow in many mammals during Pleistocene and Holocene periods (Cheylan 1991). Regarding the brown hare in general, there was some fragmentation, but definitely existing gene flow on longer geographical distances. The Anatolian populations were not genetically different in comparison with European ones and actually they are genetically similar to the continental Greek populations. Many alleles of Anatolian populations were not found in the European ones. Phylogeographic analysis based on allozyme variability revealed close relations, without a strong internal differentiation between Anatolian and European brown hares (Sert et al. 2005).

The studies on brown hares from Central, South Eastern Europe, also England and Anatolia, employing a large number

of allozymic loci (Hartl et al. 1990, 1992, 1993, 1994; Suchentrunk et al. 2000, 2001, 2003; Sert et al. 2005) showed variety of rare or regionally distributed alleles and a tendency for decreasing of genetic diversity, from Anatolia toward South East Europe to Central Europe and England (Vapa et al. 2007). Despite the relatively small differences between the gene pools on a long geographical distance, distinct regionally limited alleles, appearing with a low frequency, prove genetic variation of the gene pool on a small geographic scale (Sert et al. 2005). Liver samples of 33 hares, from 8 regions in Vojvodina area (Serbia) were used for the allozyme variability analysis using the same methodology, like the aforementioned researches for Austria, Bulgaria, Greece and Anatolia. Total 40 loci were analyzed and the results for Bulgaria – 157 hares (Suchentrunk et al. 2000) and Austria – 469 hares (Hartl et al. 1993), are compared (Table 4). The percent of polymorphic loci of Serbian hares in 7 loci was 17.5 %, which stays within the range for the Bulgarian (12.5–17.5 %) and Austrian ones (10–20 %). The same trend was found for the

mean number and frequencies of alleles. There were 7 alleles found in Bulgarian and Austrian populations not occurring in Serbian ones.

The heterozygosity (expected and observed) in brown hares from Serbia was slightly higher than in Austrian populations and nearly in the same range as the Bulgarian ones. The Serbian populations were genetically slightly closer to the Austrian than to Bulgarian ones, which were the most distant to the Austrian populations. Generally, Serbian brown hare populations were very close to Bulgarian and Austrian ones. (Vapa et al. 2007). The relatively low values of absolute genetic differentiation between Vojvodina (Serbia) and the populations of Central Europe (Austria) and South East Balkans (Bulgaria) present a slight genetic divergence in general or more precisely, a small genetic differentiation. There were no new alleles discovered in brown hares from Serbia (probably due to the small number of samples, $n=33$) and the most commonly occurring alleles were common for all three countries (Vapa et al. 2007). This research confirms the results obtained by Djan et al. (2004).

Another study on the genetic diversity in 4 regions in Vojvodina (Serbia) based on allozyme markers (Vapa et al. 2002) revealed high values for heterozygosity and polymorphism. This could be a consequence of smaller number of animals analyzed in this study, and smaller number of hypothetical structural loci, which enables detection of more alleles. The percent of polymorphic loci in different populations ranged from 11.76 % to 35.29 %. The overall rate of polymorphism (99 % criterion, 17 loci considered) amounted to 41.17 %.

Table 4. Indices of genetic variability in brown hare populations from Vojvodina (Serbia), Bulgaria and Austria (Vapa et al. 2007).

| Genetic indices | Vojvodina (Serbia) | Bulgaria | Austria |
|-----------------|--------------------|-------------|-------------|
| N | 1 | 8 | 20 |
| H_o | 0.042 | 0.032–0.056 | 0.027–0.041 |
| H_e | 0.043 | 0.033–0.058 | 0.028–0.042 |
| P | 17.5 | 12.50–17.50 | 10.00–20.00 |
| A | 1.20 | 1.17–1.25 | 1.10–1.23 |

Note: N – number of populations, H_o – observed heterozygosity, H_e – expected heterozygosity, P – percent of polymorphic loci (criterion 99 %), A – mean number of alleles per locus.

Mean observed heterozygosity in hare populations from Vojvodina was $H_o=0.070$, and mean expected $H_e=0.075$ (Table 5). The average number of alleles per locus was 1.35 (Vapa et al. 2002).

Table 5. Comparison of genetic variability in Austrian, Bulgarian, Polish and Serbian brown hare population (Vapa et al. 2002).

| Genetic indices | Austria | Bulgaria | Poland | Serbia |
|----------------------|---------|----------|--------|--------|
| <i>N</i> | 20 | 8 | 1 | 4 |
| <i>H_o</i> | 0,03 | 0,03 | – | 0,07 |
| <i>H_e</i> | 0,027 | 0,035 | 0,047 | 0,075 |
| <i>P</i> | 0,11 | 0,12 | 0,18 | 0,23 |
| <i>A</i> | 1,13 | 1,17 | – | 1,35 |

Note: *N* – number of populations, *H_o* – mean observed heterozygosity, *H_e* – mean expected heterozygosity, *P* – percent of polymorphic loci (99 % criterion), *A* – mean number of alleles per locus.

Other research (Djan et al. 2004) for allozyme variability in 31 loci was performed on livers of 60 individuals from 15 regions in Vojvodina (Serbia). The result showed that for Serbia the observed heterozygosity was 0.042, compared to Bulgaria 0.030 and Austria 0.030. Expected heterozygosity was higher in Serbian populations – 0.062 than in Bulgarian and Austrian ones – 0.035 and 0.027, respectively (Suchentrunk et al. 2000). The percent of polymorphic loci of Serbian brown hare ranged from 0 to 12.9 % with average 8.39 % as for Bulgaria it was 12 % and Austria 10.7 %. Independently of the fact that heterozygosity and polymorphism were high for Serbian hares, the allozymic variability variety was low, which correspondent with the data about east parts of Austria (Hartl et al. 1993), but

lower compared to Bulgaria (Suchentrunk et al. 2000).

Genetic Diversity and Differentiation of Brown Hares Based on RAPD Analyses

The second genetic study including individuals from Bulgaria employed a new class of markers – Random Amplified Polymorphic DNA (RAPD) (Mamuris et al. 2002). Since in Greece it is a practice to import brown hares from different countries, the study of Mamuris et al (2002) included besides 187 Greek hares, individuals from Austria ($n=10$), Poland ($n=10$), Germany ($n=46$), France ($n=21$) and Bulgaria ($n=42$), total 129. According to Mamuris (2002) one recent import from Bulgaria to Greece included 42 brown hares, which were raised in a farm for certain period and then released. Genetic samples were taken before their release in Greece, so Bulgaria was represented by these 42 individuals. The research was done also on samples on wild populations from the continental part of Greece ($n=149$), as 24 hare from them were raised in farms and 14 were semi free from wild populations, thus resulting in a total of 316 brown hares (Table 6). Bulgarian populations had high values on heterozygosity compared with those from Central Europe and a bit lower than the populations from Greece. These facts confirmed the results of Suchentrunk et al. (2003).

The result showed also that the Greek and Bulgarian populations are genetically closer between each other and in the same time divergent from Central Europe (Poland, Austria, Germany and France). Also genetically closer were the three Central European populations.

Table 6. Genetic diversity based on RAPD markers (Mamuris et al. 2002).

| Genetic indices | Zalogo | Vradeto | Pyrra | Spilia | Elas-sona | Veles-tino | Farm 1 | In enclosure | Austria/Poland | Germany | Fran-ce | Bul-garia |
|-----------------|--------|---------|-------|--------|-----------|------------|--------|--------------|----------------|---------|---------|-----------|
| <i>N</i> | 26 | 21 | 36 | 23 | 20 | 23 | 24 | 14 | 20 | 46 | 21 | 42 |
| <i>He</i> | 0.269 | 0.255 | 0.267 | 0.264 | 0.262 | 0.272 | 0.202 | 0.200 | 0.236 | 0.238 | 0.240 | 0.258 |
| <i>D</i> | 0.022 | 0.019 | 0.021 | 0.015 | 0.019 | 0.025 | 0.078 | 0.077 | 0.083 | 0.081 | 0.081 | – |

Note: *N* – number of individuals, *H* – expected heterozygosity, *D* – Nei's Genetic Distance between Bulgaria and 11 populations of brown hare based on RAPD analysis.

Genetic Diversity and Differentiation of Brown Hares Based on Microsatellite Variability

The third and last study on Bulgarian brown hares at individual level was done by Estonba et al. (2006). The study included total 100 hares belonging to three species of genus *Lepus*: *L. europaeus* – 39 from Iberian Peninsula and 31 from Bulgaria (Plovdiv – 26, Pazardjik – 4 and Karlovo – 1), *L. castroviejoii* (*n*=11), and *L. granatensis* (*n*=19). Genetic diversity and differentiation were documented in six microsatellite loci. The results showed that

genetic diversity in the two populations of *L. europaeus* from Iberian and Balkan Peninsula was similar ($p > 0.061$; Student *t*-test), but there were some differences regarding the distribution of allele frequencies (Table 7). This heterogeneity demonstrates some restrictions on nuclear gene flow between these two populations, which we can expect because of their geographical isolation. This also matches the result from Mamuris et al. (2002). Iberian population clearly group with Bulgarian ones, independently from the big distance between them, compared with the other two species in this research. As a main

Table 7. Genetic diversity based on microsatellite markers (Estonba et al. 2006).

| Loci | <i>L. europaeus</i> (Iberia) | | | <i>L. europaeus</i> (Bulgaria) | | |
|-------------|------------------------------|-----------|-----------|--------------------------------|-----------|-----------|
| | <i>MNA</i> | <i>Ho</i> | <i>He</i> | <i>MNA</i> | <i>Ho</i> | <i>He</i> |
| SOL8 | 6 | 0.615 | 0.594 | 8 | 0.806 | 0.794 |
| SAT2 | 11 | 0.579 | 0.587 | 16 | 0.833 | 0.851 |
| SAT8 | 3 | 0.231 | 0.363 | 4 | 0.742 | 0.535 |
| SOL33 | 4 | 0.243 | 0.225 | 4 | 0.414 | 0.440 |
| SAT12 | 8 | 0.821 | 0.766 | 7 | 0.806 | 0.841 |
| SAT5 | 7 | 0.424 | 0.821 | 9 | 0.263 | 0.707 |
| Mean | 6.5 | 0.486 | 0.559 | 8.0 | 0.644 | 0.695 |
| <i>S.D.</i> | 2.9 | 0.230 | 0.230 | 4.4 | 0.243 | 0.171 |

Note: *MNA* – mean number of alleles, *Ho* – observed heterozygosity, *He* – expected heterozygosity, *S.D.* – standard deviation.

conclusion the authors pointed that the geographical separation on these two populations should be kept and they should be managed as independent units. Local adapted population has to be preserved, which will lead to protection of the local genotypes. These results have direct influence on future restocking programs.

Genetic diversity was analyzed with microsatellites markers in 11 loci of 294 brown hares from genus *Lepus* from 18 different regions from North and South Africa, North and South Israel, Anatolia and Europe (Ben Slimen

et al. 2008). Three populations from Bulgaria were included in this study – Yambol, Vratsa and Pleven, and the samples were the same as in the study of Suchentrunk et al. (2000). The variance of observed heterozygosity for all populations was between 0.45 and 0.63 as for Bulgarian ones the values are: Yambol – 0.56, Vratsa – 0.54 and Pleven – 0.48 (Table 8). Expected heterozygosity for the three Bulgarian populations was one of the highest of all. The mean number of alleles per locus was 14.7, as the variation was between 6 and 34. Yambol was one of 7 populations which were in Hardy-Weinberg equilibrium. Genetic diversity decreased from South Africa, North Africa, Israel, Anatolia, the Balkans, central Europe to NW Europe (Ben Slimen et al. 2008).

Table 8. Genetic diversity found by Ben Slimen et al. (2008).

| Genetic indices | Yambol | Vratsa | Pleven | All 18 regions |
|-----------------|--------|--------|--------|----------------|
| <i>Ho</i> | 0.56 | 0.54 | 0.48 | 0.45–0.63 |
| <i>He</i> | 0.60 | 0.68 | 0.59 | 0.48–0.71 |
| <i>A</i> | 4.64 | 7.09 | 5.36 | 3.90–7.73 |

Note: *Ho* – observed heterozygosity, *He* – expected heterozygosity, *A* – mean number of alleles per locus.

In total 710 individuals belonging to 6 hare species which are occurring Europe and North Africa were studied. Concerning *L. europaeus* 323 were from Italy, Hungary, Romania, Austria, Bulgaria, Greece and Uruguay. Bulgarian samples have been taken from Estonba et al. (2006) and microsatellites marker were used in 13 loci. The thirteen microsatellite loci showed 97.44 % polymorphic loci and an average number of alleles of 6.8 for each

locus (Mengoni 2011). The mean number of alleles for *L. europaeus* for all loci was 13, expected heterozygosity – 0.69 and the observed one – 0.57.

Conclusions

The Bulgarian brown hare samples were so far subject to three different studies of genetic diversity, using allozyme, RAPD and microsatellite markers. The study employing allozyme markers was the most extensive one, and the results were compared with results from Central Europe (Austria). Also, the information from this study served as a base for comparison between many other following researches from South-East Europe (Greece and Serbia) and Asia Minor (Turkey – Anatolia). The other two studies (comparison of Bulgarian populations with Iberian and Greek ones) were done using microsatellite and RAPD markers and they confirmed the earlier results, obtained with allozyme markers. Genetic diversity decreased from South Africa to North West Europe. For the Middle East, the Balkans and Central Europe it had its highest values in Anatolian populations, followed by Greek, Bulgarian, Serbian and finally, the Austrian ones. The genetic similarities among the regions follow the same order. Hypothetically, it was due to continuous hare presence in Anatolia, Greece and probably other regions on the Balkans during the Pleistocene, when the unfavorable climate conditions did not provide habitats good enough in most of the northern territories. The hare populations of Central Europe could not receive gene flow from Southeastern Europe, including Bulgaria, which could not receive genes from Asia Minor or the North East side of Black Sea. The results also confirmed

that the separate populations in Bulgaria are very similar to each other, forming a relatively homogeneous gene pool, which is poorly differentiated from other regions in Europe and Asia Minor. There was a fragmentation of a small geographical scale, from different regionally limited alleles, appearing with a low frequency and decreased, but definitely gene flow exists among longer geographical distances. The hypothesis about potential gene flow from North Greece towards the region of Sandanski (Bulgaria) was denied. There was no negative impact on the genetic diversity caused by decrease of population size, occurred for a long periods of time, as it was the case in Greece.

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References

BEN SLIMEN H., SUCHENTRUNK F., STAMATIS C., MAMURIS Z., SERT H., ALVES P.C., KRYGER U., SHAHIN A. B., BEN AMMAR ELGAAIED A. 2008. Population genetics of cape and brown hares (*Lepus capensis* and *L. europaeus*): a test of Petter's hypothesis of conspecificity. *Biochemical Systematics and Ecology* 36: 22–39.

CHEYLAN G. 1991. Patterns of Pleistocene turnover, current distribution and speciation among Mediterranean mammals. In: R.H. Groves, F. Di Castri. *Biogeography of Mediterranean Invasions*. Cambridge University Press, Cambridge: 227–262.

CORBET G.B. 1986. Relationships and origins of the European lagomorphs. *Mammal Review* 16: 105–110.

DJAN M., VAPA L., OBREHT D., VAPA M., SELMIC V. 2004. On gene pool divergence of the brown hare (*Lepus europaeus* Pallas) in Vojvodina. *Natural Sciences, Matica Srpska, Novi Sad* 107: 13–20.

ESTONBA A., SOLIS A., IRIONDO M., SANZ M., MARIA J., PEREZ S., MARKOV G., PALACIOS F. 2006. The genetic distinctiveness of the three Iberian hare species *Lepus europaeus*, *L. granatensis*, and *L. castroviejo*. *Mammalian Biology* 71(1): 52–59.

HARTL G.B., MARKOWSKI J., KOVACS G., GRILLITSCH M., WILLING R. 1990. Biochemical variation and differentiation in the brown hare (*Lepus europaeus*) of Central Europe. *Zeitschrift für Säugetierk* 55: 186–193.

HARTL G.B., MARKOWSKI J., SWIATECKI A., JANISZEWSKI T., WILLING R. 1992. Genetic diversity in the Polish brown hare *Lepus europaeus* Pallas, 1778: implications for conservation and management. *Acta Theriologica* 37: 15–25.

HARTL G.B., SUCHENTRUNK F., NADLINGER K., WILLING R. 1993. An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA. *Acta Theriologica* 38(2): 33–57.

HARTL G.B., WILLING R., NADLINGER K. 1994. Allozymes in mammalian population genetics and systematics: Indicative function of a marker system reconsidered. In: Schierwater B., Streit B., Wagner G. P., DeSalle R. (eds). *Molecular Ecology and Evolution: Approaches and Applications*, Birkhauser Verlag, Basel, Switzerland: 299–310.

MAMURIS Z., SFUGARIS A.I., STAMATIS C. 2001. Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA PCR-RFLP analysis: implications for conserving genetic diversity. *Biological Conservation* 101: 187–196.

MAMURIS Z., SFUGARIS A.I., STAMATIS C., SUCHENTRUNK F. 2002. Genetic structure of Greek brown hare (*Lepus europaeus*) populations based on the Random Amplified Polymorphic

DNA (RAPD) method. *Biochemical Genetics* 40: 323–338.

MENGGONI CH. 2011. Phylogeny and genetic diversity of Italian species of hares (genus *Lepus*). PhD thesis. Bologna University, 47 p.

SERT H., SUCHENTRUNK F., ERDOGAN A. 2005. Genetic diversity within Anatolian brown hares (*Lepus europaeus* Pallas, 1778) and differentiation among Anatolian and European populations. *Mammalian Biology* 70: 171–86.

SFOUGARIS A., PAPAGEORGIOU N., GIANNAKOPOULOS A., GOUMAS H., PAPAEVANGELOU E., ANNI A. 1999. Distribution, populations and habitat of the European hare (*Lepus europaeus* Pallas, 1778) in central and western Greece. In: Thomaidis C., Kypridimos N. (eds). Proceedings of 24th Congress of International Union of Game Biology: Agriculture–Forestry–Game: Integrating Wildlife in Land Management, Sept. 20–24. 1999. Thessaloniki, Greece: 423–430.

SMITH R.K., JENNINGS N.V., HARRIS S. 2005. A quantitative analysis of the abundance and demography of European hares *Lepus europaeus* in relation to habitat type, intensity of agriculture and climate. *Mammal Review* 35: 1–24.

SMITH A.T., JOHNSTON C.H. 2008. *Lepus europaeus*. The IUCN Red List of Threatened Species. Version 2014.2. Available: www.iucnredlist.org (Accessed on 05 September 2014).

STRZAŁA T., KOSOWSKA B., BRZEZINSKA K., MOSKA M. 2006. Charakterystyka genetyczna zajęcy (*Lepus* sp.) w Eurazji [Genetic characteristics of hares (*Lepus* sp.) in Eurasia.] *Acta Scientiarum Polonorum, Medicina Veterinaria* 5(1): 25–31. (In Polish with English summary).

SUCHENTRUNK F., POLSTER K., GIACOMETTI M., RATTI P.C.G., THULIN C., RUHLE C., VASILEV A.G., SLOTTA-BACHMAYR L. 1999. Spatial partitioning of allozyme variability in European mountain

hares (*Lepus timidus*): Gene pool divergence across a disjunct distributional range? *Zeitschrift für Säugetierkunde* 64: 1–11.

SUCHENTRUNK F., MIHAILOV CH., MARKOV G., HAIDEN A. 2000. Population genetic of Bulgarian brown hare *Lepus europaeus*: Allozymic diversity at zoogeographical crossroads. *Acta Theriologica* 45(1): 1–12.

SUCHENTRUNK F., JASCHKE C., HAIDEN A. 2001. Little allozyme and mtDNA variability in brown hares (*Lepus europaeus*) from New Zealand and Britain – A legacy of bottlenecks? *Mammalian Biology* 66: 48–59.

SUCHENTRUNK F., MAMURIS Z., SFOUGARIS A.I., STAMATIS C. 2003. Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. *Biochemical Genetics* 41: 127–140.

TATATRUCH F., ONDERSCHEKA K., BOTEV N., NINOV N., MIHAILOV Ch. 1996. Die Schwermetallbelastung der freilebenden Wildtiere in Bulgarien. XII IUGB Congress „The Game and the Man“, Sofia, Bulgaria, Sept. 1995: 432–438.

VAPA L., OBREHT D., VAPA M., SELMIC V. 2002. Genetic variability in brown hare (*Lepus europaeus*) populations in Yugoslavia. *European Journal of Wildlife Research* 48: 261–266.

VAPA L., DJAN M., OBREHT D., HAMMER S., SUCHENTRUNK F. 2007. Allozyme variability of brown hares (*Lepus europaeus*) from the Vojvodina (Serbia), compared to central and southeastern European populations. *Acta Zoologica* 53(1): 75–87.

VAUGHAN N., LUCAS E.A., HARRIS S., WHITE P.C.L. 2003. Habitat associations of European hares *Lepus europaeus* in England and Wales: Implications for farmland management. *Journal of Applied Ecology* 10(1): 163–175.

WILSON D.E., REEDER D.M. 2005. *Mammal Species of the World. A Taxonomic and Geographic Reference* (3rd ed), Johns Hopkins University Press, 2142 p.