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# Genetic Diversity of the Red Deer (*Cervus elphus* L.) in Hungary Revealed by Cytochrome *b* Gene

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**Abstract:** The genetic diversity of Red Deer (*Cervus elaphus*) in Hungary was studied using a full-length cytochrome *b* gene (1140 bp). The haplotype structure of this species was evaluated in the three main partially geographically separated populations in this country. Throughout the country, the genetic diversity of the Red Deer was represented by individuals belonging to all the three distinct mitochondrial lineages of this species in Europe, i.e. western, eastern and Sardinian/Bulgarian. The presence of all the three mtDNA lineages in the Red Deer populations in Hungary implied the existence of an overlapping zone among them. The genetic distinction between Red Deer populations inhabiting this zone showed the highest levels of dissimilarity of the northeast population. The recorded population genetic peculiarities and the geographical variability of cytochrome *b* gene haplotypes in Hungarian Red Deer as well as clarification of its phylogenetic relationships to other genetically characterised populations allowed us to identify its intraspecific population differences in Hungary.

**Keywords:** *Cervus elaphus*, Cytochrome *b*, genetic diversity, Hungary, Red deer

## Introduction

The Red Deer (*Cervus elaphus* L.) is a widespread ungulate species in Europe with remarkable economic and cultural values (Milner *et al*. 2006). Its populations are increasing both in Central and in Western Europe (Burbaite, Csányi 2010) as well as in Hungary (Rivrud *et al*. 2012). The number of the Red Deer in Hungary increased from 16 733 individuals in 1960 to 94 135 individuals in 2011. Nowadays, this species can be found in a large part of the territory of the country. It is absent only in some lowland regions of the central part of Hungary (Szemethy *et al*. 2007, Csányi, Lehoczki 2010).

In the light of the genetic studies of the Red deer carried out so far in Europe, the species can potentially reveal high genetic variability in the territory of Hungary (for details, see Martínez *et al*. 2002, Pérez-Espona *et al*. 2008, Skog *et al*. 2009, Zachos, Hartl 2011, Fernández-García *et al.* 2013).

In addition to a number of internal factors, the genetic diversity of the Red Deer is also determined by a variety of external factors (for details, see Pérez-Espona *et al*. 2008). Among them, it is the landscape features (Manel *et al*. 2003) that could affect the dispersal ability of animals, respectively the gene flow between populations of animals and their genetic structure. Natural barriers such as rivers (Cullinghamet *et al*. 2009, Frantz *et al.* 2010) and man-made barriers (Eppset *et al*. 2005, Riley *et al*. 2006, Frantz *et al.* 2010) such as roads and areas under human impact are particularly important for the formation of population genetic diversity of the species in a given area.

The mitochondrial DNA (mtDNA) is a highly sensitive genetic marker, which is suitable for the examination of population structures within a species (Sunnucks 2000). Phylogenetic and phylogeographic studies of the Red Deer based on mtDNA (Cyt *b*) (Zachos, Hartl 2011, Niedziałkowska *et al*. 2011, Markov *et al.* 2012) constitute a good starting base for assessing the genetic structure and its variation in each Red Deer population that we begin to investigate. In this study, we examined the genetic structure of the Red Deer in Hungary, using a cytochrome *b* gene genetic analysis. Our aims were to: (i) identify the main mtDNA (Cyt *b*) haplogroups in Red Deer in Hungary; (ii) describe the phylogenetic relationships between Hungarian and other European cytochrome *b* gene lineages; and (iii) construct a picture of their genetic dissimilarity on the basis of the observed patterns of mtDNA (Cyt *b*) genetic diversity within and among the studied Hungarian Red Deer populations.

## Material and Methods

The presence of a well-expressed river system in Hungary and man-made barriers such as roads and cultivated areas pre-determined the three studied geographic Red Deer populations, located in the west-east direction as follows: Southwest population (Pop 1) – from the west state border along the Danube River valley; Central population (Pop 2) – from the Danube River valley to the river system of the Zagyva and Tisza; Northeast population (Pop 3) – from the river system of the Zagyva and Tisza to the country's east border. Tissue samples (liver) of forty-two free living Red Deer individuals were collected from these three localities in Hungary: 21 specimens from Southwest (Pop 1), 11 specimens from Central (Pop 2) and 10 specimens from Northeast (Pop 3) part of the country (Fig. 1).

To assess the genetic relationship of the Hungarian Red Deer with some other geographically separated populations of *C. elaphus* in its European range, other 17 individuals originating from Belarus (n=4), Ukraine (n=6), Crimea (n=5), Carpathian Mountains (n=1), Kaliningrad, Russia (n=4), and Bulgaria (n=3) were studied. Furthermore, 11 GenBank sequences of cytochrome *b* of specimens from Europe and Africa were included in the analysis (Table 1).

DNA was isolated from muscle or liver tissue by a Diatom®DNA Prep reagent kit (Russia). PCR was carried out using primers for complete sequence of cytochrome *b* described by Kocher *et al.* (1989). The whole cytochrome *b* sequences were amplified according to a PCR-protocol for the above-mentioned

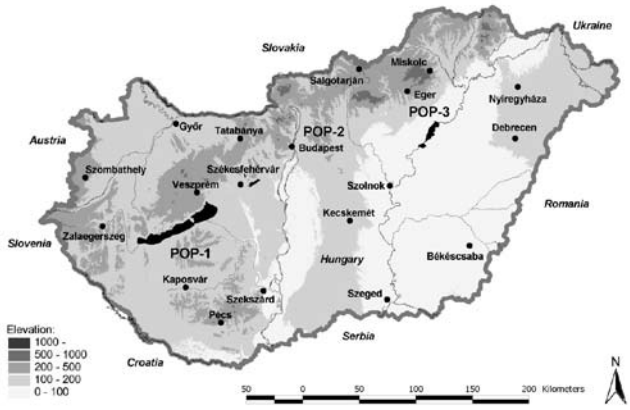


**Fig. 1.** Sampling areas of the Red deer (*Cervus elaphus* L.) in Hungary: Pop 1 – southwest; Pop 2 – central, and Pop 3 – northeast part of the country

primers (denaturation at 95°C for 4 min; 30 cycles at 95°C for 30 s, 65°C for 30 s, 72°C for 1 min; 72°C for 5 min). The DNA sequences were identified using an ABI PRISM® BigDyeTM reagent kit Terminator v. 3.1 on an ABI PRISM 3100 Avant automatic DNA sequencer (USA). All the sequences were aligned manually using the BioEdit program (Hall 1999). The nucleotide diversity and distance between haplogroups were revealed using MEGA 5.0 (Tamura *et al*. 2011). A Median network of haplotypes was built in the Network 4.111 program (Bandelt *et al*. 1999). All distances were calculated in a MEGA 5.0 program using the Kimura 2-parameter model (Kimura *et al*. 1980). PAUP* 151 4.0b10 (Swofford 2002) was used to reconstruct the maximum parsimony tree. The significance of the tested phylogenetic relationship was evaluated by a Bootstrap analysis with 1000 replicates (Felsenstein 1985).

## Results and Discussion

The investigated data set included 70 cytochrome *b* sequences. A total of 40 (3.5%) parsimony-informative sites were identified. The nucleotide diversity of the complete set was 1.2 % (according to a p-distance model). The estimated transition/transversion bias (R) was 4.32. The nucleotide ($\pi$) and haplotype (H) diversity of the Hungarian Red Deer populations are shown in Fig. 2.

The highest nucleotide diversity was detected in Pop 3 (Fig. 2A) originating from the northeast part of Hungary. This diversity was 25.0% greater than that in Pop 2 from central Hungary and 12.5% greater than the nucleotide diversity in Pop 1 from southwest Hungary. The highest haplotype diversity was found in Pop 1 (Fig. 2B) and was more or less similar to the haplotype diversity in Pop 2 (relative

**Table 1.** Red deer sampling localities and type of their mitochondrial DNA haplotype lineage classified as belonging to any of the 3 different mitochondrial lineages of *Cervus elaphus* in Europe – western lineage (A), eastern lineage (C) (Ludt *et al.* 2004; Skog *et al*. 2009; Niedziałkowska *et al.* 2011) and a third lineage (B) confined to Mediterranean island Sardinia (Italy) (Zachos, Hartl 2011) and Bulgaria (Markov *et al*. 2012). In the table cells the specimen numbers of the studied red deer are recorded, as registered in the Molecular Diagnostics Methods Room of Institute of ecology and evolution, Russian Academy of Sciences or in GenBank; this specimens characterize the population haplotype identification of the species in different European countries; the same numbers are presented in the figures

| **Origin of red deer specimens** | **Haplotype lineage** | | |
|---|---|---|---|
| | **Haplotype A** | **Haplotype B** | **Haplotype C** |
| Red deer from Hungary | | | |
| POP1<br>(Southwest part of the country) | | 380HU; 382HU;<br>387HU; 2378 | 386HU; 392HU; 2353; 2354; 2355;<br>2356;2357; 2358; 2359; 2360;2361;<br>2362; 2363; 2364<br>2365; 2366 ;2367 |
| POP2<br>(Central part of the country) | 28H; 30H | | 4H; 14H; 16H; 17H; 19H; 21H; 22H;<br>23H; 31H |
| POP3<br>(Northeast part of the country) | 2231; 2232; 2233;<br>2234; 2240 | | 2235; 2236; 2237; 2238; 2239 |
| Red deer from its European areal outside Hungary | | | |
| Country | Haplotype A | Haplotype B | Haplotype C |
| Belarus<br>(Brest region) | 7; 8 | | |
| Belarus<br>(Mogilev region) | 1039; 1041 | | |
| Ukraine<br>(Crimea peninsula) | 43; 72; 73; 74; 1033 | | |
| Ukraine<br>(Carpathian mountains) | 70 | | |
| Russia<br>(Kaliningrad region) | 75;76;77';78 | | |
| Bulgaria<br>(South part of the country) | | 11BLG; 2BLG | 8Blg |
| GenBank Sequences | | | |
| Country | Haplotype A | Haplotype B | Haplotype C |
| Turkey | | | AY118197 |
| Austria | | | AY044857 |
| Romania | | | AY070225 |
| Poland | AY044860 | | |
| Germany | AY044858 | | |
| Spain | AY044859 | | |
| Norway | AY070226 | | |
| Scotland | AB021099 | | |
| Tunisia | | AY118198; AY070222 | |
| Italy (Sardinia) | | AY244489 | |

difference 4.5%) but much greater than the haplotype diversity in Pop 3 (relative difference 24.7%). The relative difference between the haplotype diversity in Pop 2 and Pop 3 was 18.3%.

The estimated number of haplotypes in the analysed 42 individuals was 36. The network of haplotypes (Fig. 3) was derived taking into account 179 mutations. The cytochrome *b* gene variety of the Red Deer in Hungary revealed during the present study showed the existence of 3 distinct mitochondrial lineages: Western lineage (A), Eastern lineage (C), typical of the species in Europe (Ludt *et al.* 2004, Skog *et al*. 2009, Niedziałkowska *et al.* 2011), and a third lineage (B) confined to North Africa, the Mediterranean island of Sardinia, Italy (Zachos, Hartl 2011) and Bulgaria (Markov *et al*. 2012).

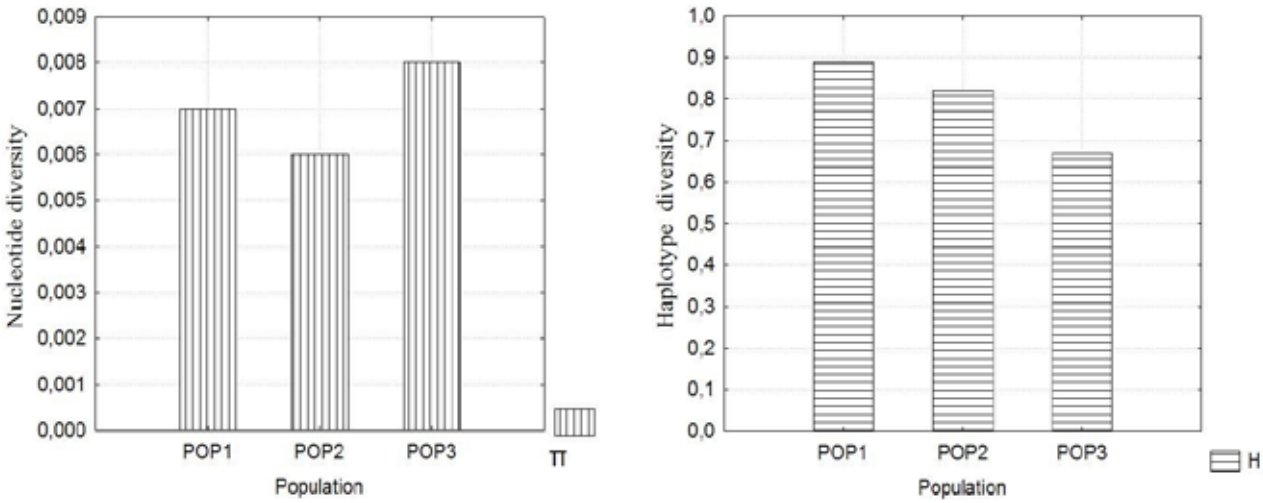


**Fig. 2.** The nucleotide (π) (A) and haplotype (H) diversity (B) of the Hungarian Red deer populations. Origin of populations: POP-1 – southwest; POP-2 – central, and POP-3 – northeast part of the country

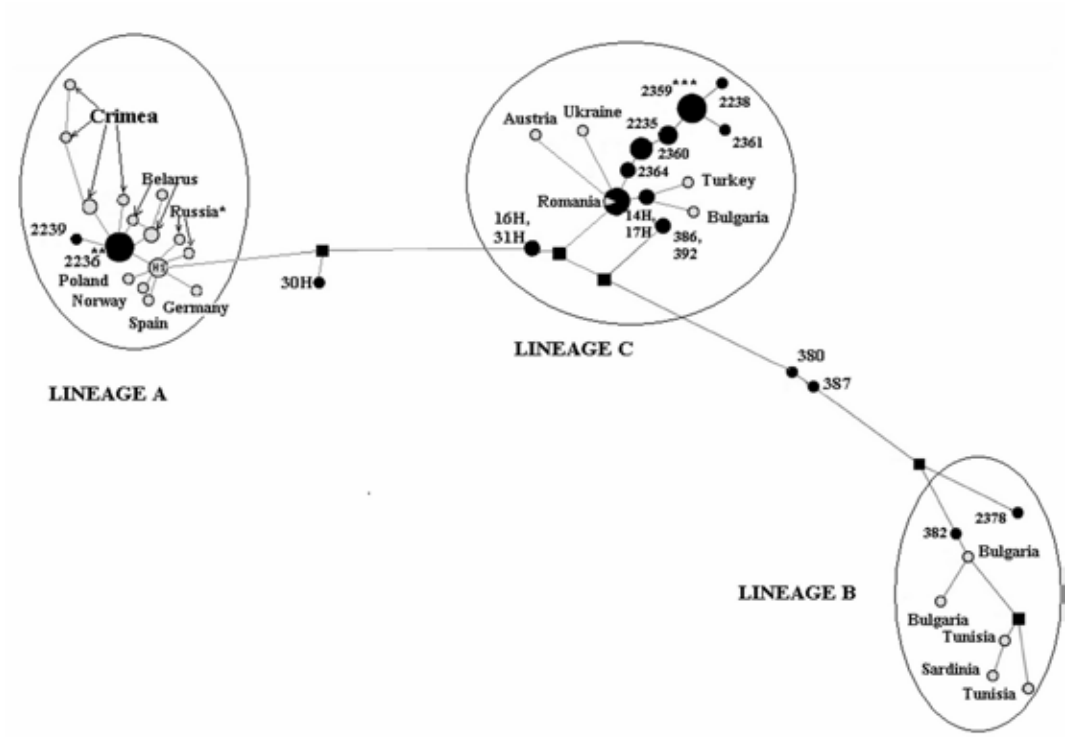


**Fig. 3.** Median-joining network of investigated seventy Red Deer mtDNA cytochrome *b* haplotypes from various countries in Europe and Africa. Length of branches corresponds to the number of mutational steps between haplotypes. The Red Deer from Hungary is shown in black, while its geographical origin is shown in Table 1. The symbol size reflects the number of included individuals. Some individuals have identical haplotypes and have the following symbols H1: AB021099 (Scotland), 76 and 77 (Russia, Kaliningrad Region); 4H, 19H, 21H, 22H, 23H (Romania); 75 and 78 (Russia, Kaliningrad Region); 2236** (2231, 2232, 2233, 2234, 2240 and 28H); 2360: (2355, 2356 and 2363); 2235: (2237, 2353) and 2359: (2354, 2357, 2358, 2362 and 2366)

In the present study, the "haplotypes lineage A" was represented by the Red Deer from Spain, Germany, Norway, Poland, Scotland and Hungary; the „haplotypes lineage C" was presen by the specimens from Austria, Romania, Turkey, Ukraine (Carpathian Mountains) and Hungary; and the „haplotypes lineage B" included samples from Sardinia, Tunisia, Bulgaria and Hungary. The following haplotypes that occupied an intermediate position between the main three haplogroups were also revealed in some individuals from the Hungarian populations of the Red Deer: haplotype 30H (between "A" and "C")

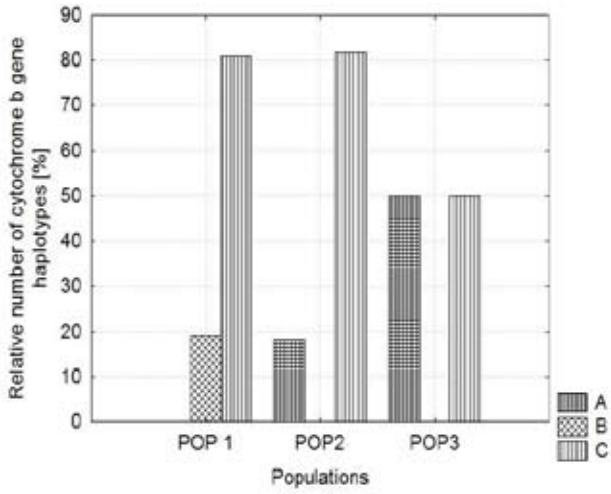


**Fig. 4.** The frequency of the recorded cytochrome *b* haplotypes in the Hungarian populations corresponding to the three mitochondrial lineages of the Red Deer in Europe. Origin of populations: Pop 1 – southwest; Pop 2 – central and Pop 3 – northeast part of the country. Mitochondrial lineages of the Red Deer in Europe: A – western lineage; C –eastern lineage; and B – southern lineage

and haplotypes 380 and 387 (between "C" and "B").

The frequency of the recorded haplotypes in the Red Deer from Hungary, which corresponded to each of the three genetic lines, was: (i) 9.5% for line „A"; (ii) 16.7% for line "B"; and (iii) 73.8% for line "C".

The spatial distribution of the haplotype patterns within the analysed Hungarian populations revealed two groups of haplotypes in each of the studied populations:

(i) In the southwest population (Pop 1) – haplotypes, belonging to the lineages "C" and "B";

(ii) In the central population (Pop 2) – haplotypes, belonging to the lineages "A" and "C";

(iii) In the northeast population (Pop 3) - two groups of haplotypes that belonged to the lineages "A" and "C" as well.

The relative number of cytochrome *b* haplotypes belonging to any of its three distinct lineages in Hungarian Red Deer populations, is shown on Fig. 4.

For the first time in a Red Deer population from Central Europe, haplotypes of the lineage "B", characteristic of the southern part of the species range, were found in the southwest part of Hungary (Pop 1). The spatial distribution of the mtDNA haplotypes within the Red Deer in Hungary, as evaluated in regard to the geographical gradient from the northeast part to the southwest part of the country, showed the following:

(i) A relative reduction in the presence of haplotypes belonging to the western lineage (A) and a

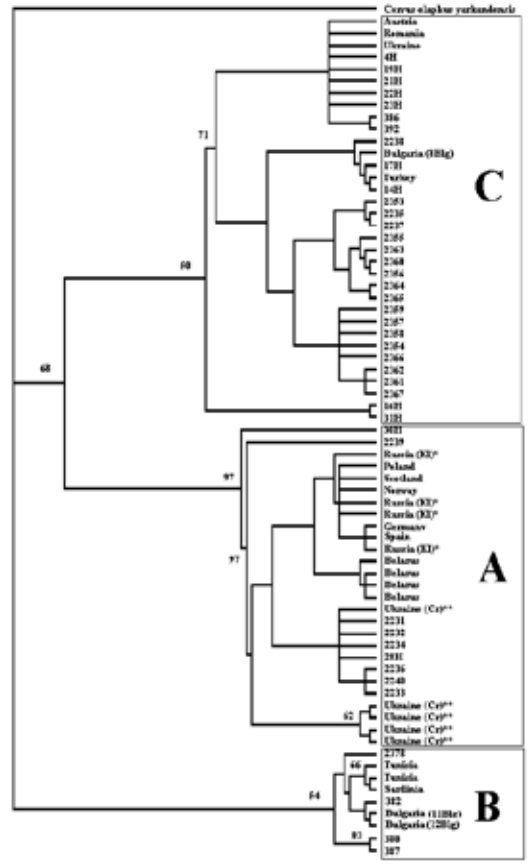


**Fig. 5.** Phylogenetic relationships of the studied Red Deer based on haplotype patterns revealed by the cytochrome *b* gene distinction. Origin of specimens and their associated haplotypes are shown in Table 1. The geographic locations of the Hungarian populations are depicted in Fig. 1. The denotation "(Kl)*" corresponds to the Kaliningrad Region of Russia, and "(Cr)**" corresponds to the Crimea

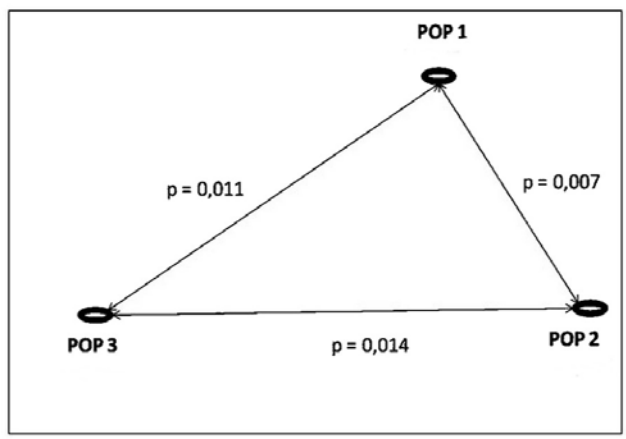


**Fig. 6.** Plot of genetic dissimilarity (p-distance) among the Red Deer populations in Hungary as found by using the mitochondrial cytochrome *b* gene as a molecular marker to assess their genetic diversity. Abbreviations correspond to: Pop 1 – population from southwest Hungary, Pop 2 – population from central Hungary and Pop 3 – population from the northeast Hungary

relative increase in the presence of haplotypes belonging to the eastern lineage (C);

(ii) A reduction in the haplotypes typical of the northeast population belonging to the western lineage (A) and their replacement with haplotypes belonging to the lineage "B" in the southwest part of the country.

The topology of the maximum parsimony tree is consistent with the results gained from the Median-joining network reconstruction algorithm. The maximum-parsimony analysis confirmed the existence of three clusters (Fig. 5). These clusters corresponded to the obtained mitochondrial lineages and each of them involved representatives of the Red Deer from the Hungarian populations.

The presence of all three mtDNA lineages in the Red Deer populations in Hungary allowed assuming the existence of an overlapping zone among them. The genetic distinction between the Red Deer populations inhabiting this zone as assessed on the basis of the obtained information about mitochondrial cytochrome *b* gene by computing genetic dissimilarity among populations and presented as a "p-distance" is shown in Fig. 6. The set of the values of "p-distance" presented the genetic differentiation among the investigated Red deer populations in Hungary and showed the highest levels of distinction in the Northeast population (Pop 3).

The described population genetic peculiarities and the geographical structure of haplotypes variability in the Red Deer in Hungary as well as the the phylogenetic relationship with other populations of this species, allows us to identify the intraspecific population differences of the Red Deer in Central Europe.

In view of the idea that the management of the Red Deer populations should take into account not only their economic value but also the fact that they are part of the biodiversity of ecosystems, the revealed spatial genetic variation within the Hungarian Red Deer becomes part of the scientific support (DiBattista 2008) for actions associated with the intense management focused on the Red Deer populations. This management should target also the conservation of the genetic diversity within the species.

The widened characteristics of population genetic structure, studied through an extended set of molecular markers (sequences of the mitochondrial DNA and microsatellite loci), which are known to be powerful tools in studies of population genetics of cervids (Zachos *et al*. 2007), will reveal the potential effects of the habitat features and natural or artificial barriers for formation of the biological diversity within the species in Europe.

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