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RESEARCH ARTICLE

Genetic diversity of the stag beetle, *Lucanus cervus* (Insecta, Coleoptera) in Romania based on ND1 mitochondrial sequences

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Abstract

Lucanus cervus (Linnaeus, 1758) is one of the largest Coleoptera species in Europe and one of the most recognizable. It is a saproxylic species living in woodlands and wooded urban habitats and spending its larval stages inside tree trunks below or near soil level, feeding on decaying wood. The stag beetle has a wide distribution covering the entire Europe up to the Ural Mountains. In this paper, we attempted to describe the diversity and genetic structure of *L. cervus* populations in Romania using the NADH mitochondrial molecular marker. We identified 36 haplotypes arranged in a star-shaped structure with a central haplotype shared by many samples and linked by one or two mutational steps with other 35, represented by 1–3 samples. This pattern is frequently attributed to populations that have undergone recent range expansion, a phenomenon which is also sustained by the mismatch analysis. The analyzed populations revealed various degrees of genetic diversity. Many populations with relatively high levels of genetic diversity were found outside the Natura 2000 Network Special Areas of Conservation, while

some populations included in protected areas proved to have low values of genetic variability. This underlines the need to review the designated protection sites for this species in Romania.

Keywords
NADH mitochondrial marker, Natura 2000 Network, stag beetle, phylogeography, spatial analyses, patterns of genetic diversity, species conservation.

Introduction

Lucanus cervus (Linnaeus, 1758) is one of the largest Coleoptera species in Europe and one of the most emblematic insect across the continent. The male is easily recognizable by its large mandibles with strong tooth-like structures, resembling deer antlers, hence its common name (Solano et al. 2016). The species is characterized by a pronounced variation in body size and color, length of mandibles, size and position of the median mandibular tooth, number of antennomeres of the terminal antennal club (Harvey et al. 2011). The mandibles are large and curved and males use them as weapons in fights with other males for feeding sites or for female attention (Lagarde et al. 2005).

The species has a wide geographical distribution in Europe, from southern Sweden to Spain, Italy and Greece and from Portugal to the Ural Mountains (Harvey et al. 2011). The stag beetle is found across different types of habitats, both natural and anthropic (cities, parks, urban areas etc.). The species has a life cycle of 4 to 7 years, spending most of it as a larva living inside tree trunks and feeding on dead or decaying wood. The species is considered an engineer of saproxylic habitats, shaping and altering the decaying wood and making it suitable for other saproxylic species. Populations of this species live predominantly in oak forests situated at lowland and medium altitudes, from sea level to 1000m, but there are also records from higher altitudes, up to 1500 m (Bardiani et al. 2017).

While *L. cervus* is widely distributed in Europe, the populations from the north and central parts of its range experienced a significant decline in recent years (Harvey et al. 2011). For this reason, the IUCN European Regional Assessment listed the species as Near Threatened (Nieto et al. 2010). The species is protected by the Bern Convention since 1979 (Luce 1996). In 2021, Méndez and Thomaes found that European populations of *L. cervus* exhibit an increasing gradient of threat, from southern countries where the species is registered as Least Concern (Romania, Hungary, Slovakia) to northern countries where it is classified as Critically Endangered (Germany) or even Extinct (Lithuania, Estonia, Denmark) (Méndez and Thomaes 2021). The species is also listed on Annexes II and IV of the Habitats Directive (CE 92/43/EEC/1993) as a species of community interest whose conservation requires the designation of special areas of conservation. The species is protected in 2578 Natura 2000 sites across Europe and in 84 sites in Romania (https://eunis.eea.europa.eu/species/Lucanus%20cervus).

In Romania, the species was extensively studied from a faunistic point of view (Procheș 1997; Chimișliu 2007; Stancă-Moise 2021). Recent papers concerning the species distribution were published by Stan (2013) and Moise et al. (2023). Studies on the population genetics of *L. cervus* in Europe are very scarce. Genetic diversity is relatively high across Europe, especially in Greece, with a decreasing trend towards the northern and western parts of its European distribution range (Cox et al. 2013; Cox et al. 2019). Local studies in Ukraine showed a high genetic diversity in some populations occurring in natural forests (Snegin 2011, 2014) and a significant decrease in diversity in regions with anthropogenic influence (Snegin et al. 2017). Another study identified signatures of a recent demographic decline among the populations in a Belgian metapopulation (Cox et al. 2020).

In the current study we examined the genetic structure of the Romanian *L. cervus* metapopulation, considering also separately the male and female patterns of genetic diversity, as we might have expected that the very marked sexual dimorphism in the species could have some effect on the genetic structure of both sexes. Given that males are much more visible than females (Katušić et al. 2017; Bardiani et al. 2017), we might expect that the selective pressure (e.g., being prayed by other animals) would be different between sexes.

Up to date, no previous information about the genetic structure of Romanian populations of *L. cervus* is available, except some summary mentions in a paper studying the species at European level (Cox et al. 2019). Thus, our research is the first extensive molecular approach to *L. cervus* phylogeography in Romania and aims to describe the genetic diversity of *L. cervus* populations in Romania using the NADH mitochondrial molecular marker.

Material and methods

Sample collection and spatial analysis of populations

The adults of *L. cervus* can be observed between the end of May and the beginning of August and feed on sap from different tree species: oak (*Quercus* sp.), beech (*Fagus* sp.), linden (*Tillia* sp.). Samples were collected from all over Romania (Fig. 1) and are represented by beetle remains (beetle carcasses found as road kill or prey leftovers), totaling 188 individuals collected from 66 localities and preserved in 96% ethanol at 4°C for molecular analyses. The samples were assigned to populations as follows: a distance matrix was computed in QGIS v.3.28 Firenze and all samples within a 3km distance were merged into a single population. The procedure is based on the findings of Rink and Sinsch (2007) that above a 3km distance stag beetle populations can be considered as isolated. This resulted in 60 populations with a sample size between 1 and 25 individuals.

We also performed a spatial analysis in QGIS v.3.28 in which we overlapped layers with the biogeographical regions and Natura 2000 system of protected areas

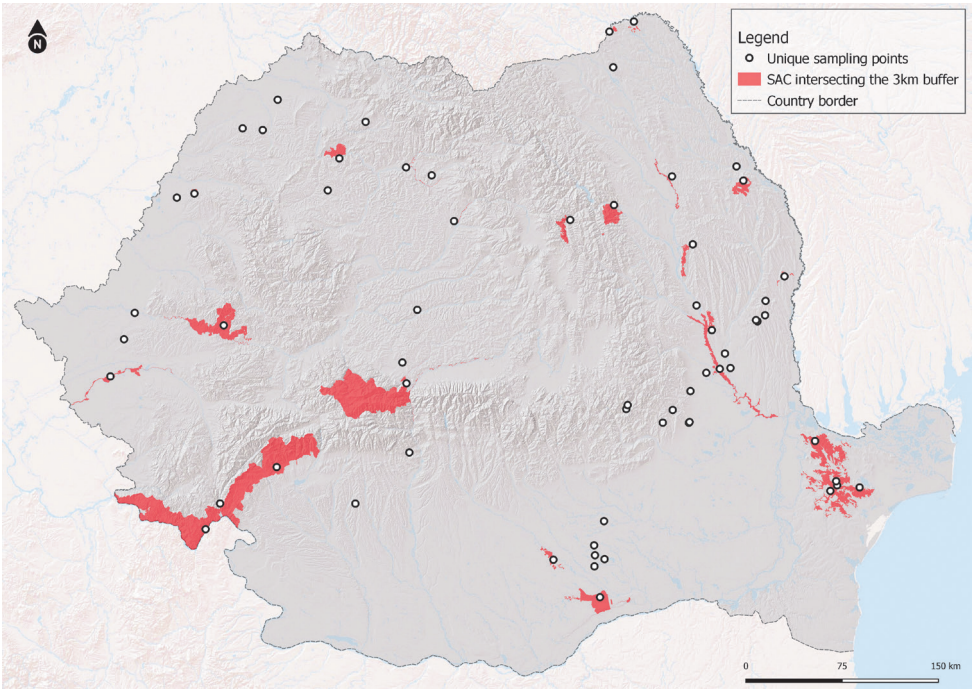

Figure 1. Geographical distribution of the stag beetle samples collected in Romania. Circles represent unique sampling points and red areas represent Natura 2000 sites where we sampled *L. cervus* populations.

for Romania in order to assess the distribution of our samples between vegetation ecoregions and in relation with the network of Natura 2000 Special Areas of Conservation (SACs). Basically, a buffer of 3 km was applied around each population to check if the dispersal distance of the species would reach a designated protected area. An intersection between the buffer and the SAC shapefiles was performed and a list of within-reach protected areas was generated. The 3 km buffer size was also chosen in accordance with Rink and Sinsch (2007). A population count for each of the five bioregions of Romania was performed.

DNA extraction and PCR amplification

Total genomic DNA was extracted from thoracic or leg muscles using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germania) according to the producer's specifications.

Polymerase chain reaction was used to selectively amplify the NADH dehydrogenase subunit 1 (ND1) gene (Hamilton et al. 2011), which has been used in other population studies of insects, such as Hemiptera (Kosovac et al. 2018), Coleoptera (Pawson et al. 2003) and butterflies (Wendt et al. 2021). ND1 is a mitochondrial gene that encodes for a subunit of complex I in the oxidative

phosphorylation system. The amplification was performed using a specific primer set (N1 J12261 and LR N12945) (Simon et al. 1994). The PCR profile was as follows: (1) an initial denaturation at 95°C for 5 minutes, followed by (2) 35 cycles of denaturation at 94°C for 30 seconds, annealing at 47°C for 45 seconds, and an extension at 72°C for 1 minute and 30 seconds, and (3) a final extension step at 72°C for 5 minutes. The amplified products were purified using FavorPrep Gel/PCR Purification Mini Kit (Favorgen, Viena, Austria) according to manufacturer's protocol. Sequencing was performed by commercial services (https://macrogen-europe.com/).

Data analyses

Sequences were edited and aligned with CodonCode Aligner v.3.7.1 (CodonCode, Dedham, Massachusetts, USA). We obtained 188 sequences that were trimmed at a length of 589 bp. The sequences were aligned using Muscle, implemented in Mega X (Kumar et al. 2018), under default options. Genetic distances were calculated using the Kimura 2 parameter model (K2P) as implemented in the same program, with 1,000 bootstrap replicates. All haplotypes obtained in this study were submitted to GenBank with accession numbers summarized in Table 1.

Analyses regarding polymorphism statistics, demographic history, Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) neutrality tests and haplotype diversity were performed in DnaSP 6.10.04 (Rozas et al. 2017). Demographic history of the Romanian *L. cervus* populations was inferred using mismatch distribution analyses. A haplotype network of our samples was generated in PopArt v.1.7 (Leigh and Bryant 2015) using a median joining algorithm (Bandelt et al. 1999). Haplotype networks are suitable to depict relationships within species and are frequently used because nodes represent ancestral haplotypes (Posada and Crandall 2001).

We also performed an isolation by distance (IBD) analysis that investigates the relationship between genetic distance (calculated as K2P and p-distance) and geographic distance among individuals or populations (Slatkin 1993). The analysis was performed in GenAlEx v.6.5 (Peakall and Smouse 2012) and is based on the assumption that genetic drift and gene flow are influenced by geographical distance, such that individuals that are geographically closer are more likely to be genetically similar than those that are far apart.

Results

Phylogenetic analyses and genetic diversity

In the entire *L. cervus* Romanian metapopulation we detected 36 haplotypes, with a haplotype diversity (Hd) of 0.461 and nucleotide diversity of 0.001. There were 35 polymorphic sites, out of which 12 were parsimony informative (Table 2). The

Table 1. ND1 haplotype distribution among sampling points and individuals. N= total number of individuals in a population; M= male; F=female; U=unknown sex, *=populations located within a Natura 2000 Site.

№	Population (Locality)	Pop code	N	Latitude	Longitude	Haplotype	Sex	GenBank accession number
1	Ardud (SM)	SM1	2	47.64354	22.86098	Hap_3	2M	OQ985060
2	Babadag (TL)*	TL1	2	44.88204	28.71148	Hap_3	1M	OQ985061
						Hap_5	1F	OQ985117
3	Bacău (BC)	BC1	1	46.2	27.15	Hap_3	1M	OQ985062
4	Bălăbăneşti (GL)	GL1	11	46.08395	27.74636	Hap_3	4M,1F	OQ985063
						Hap_16	2M	OQ985132
						Hap_17	1M	OQ985133
						Hap_18	1M	OQ985134
						Hap_19	1M	OQ985135
						Hap_21	1M	OQ985139
5	Băneasa (IF)	IF1	2	44.5311	26.0824	Hap_27	1M	OQ985147
						Hap_31	1M	OQ985154
6	Baranca (BT)*	BT1	2	48.1333	26.3166	Hap_3	1M,1F	OQ985064
7	Barcea (GL)	GL2	1	45.75404	27.47346	Hap_3	1M	OQ985065
8	Bârnova (IS)*	IS1	7	47.0655	27.6667	Hap_3	6M,1F	OQ985066
9	Bâsca Mare (BZ)	BZ1	1	45.51159	26.4365	Hap_3	1F	OQ985067
10	Beceni (BZ)	BZ2	3	45.3833	26.7833	Hap_3	2M	OQ985068
						Hap_11	1M	OQ985126
11	Beclean (BN)*	BN1	1	47.18605	24.20075	Hap_3	1M	OQ985069
12	Betfia (BH)*	BH1	2	46.9666	22.0333	Hap_3	2M	OQ985070
13	Bicaz (NT)*	NT1	1	46.8166	25.8833	Hap_3	1M	OQ985071
14	Bolintin Vale (GR)*	GR1	25	44.4335	25.6824	Hap_3	14M,3F	OQ985072
						Hap_6	1M	OQ985119
						Hap_9	1M	OQ985124
						Hap_23	1M	OQ985141
						Hap_24	1F	OQ985142
						Hap_25	1F	OQ985143
						Hap_26	1M	OQ985144
						Hap_27	1M	OQ985146
						Hap_28	1F	OQ985149
15	Brebeni (MM)	MM1	1	47.5016	23.7775	Hap_8	1U	OQ985122
16	Buciumeni (GL)*	GL3	11	46.02346	27.2983	Hap_3	5M,2F,3U	OQ985073
						Hap_20	1F	OQ985137

Table 1. (continued)

№	Population (Locality)	Pop code	N	Latitude	Longitude	Haplotype	Sex	GenBank accession number
17	București (IF)	IF2	3	44.3833	26.0833	Hap_3	3M	OQ985074
18	București (IF)	IF3	1	44.4615	26.0903	Hap_3	1F	OQ985075
19	Bursuci (VS)	VS1	2	46.2166	27.85	Hap_3	1M	OQ985076
						Hap_26	1M	OQ985145
20	Cean (SM)	SM2	1	47.43556	22.50608	Hap_29	1M	OQ985151
21	Ciucurova (TL)*	TL2	1	44.9036	28.49	Hap_3	1M	OQ985077
22	Ciucurova (TL)*	TL3	1	44.9333	28.4833	Hap_3	1F	OQ985078
23	Comana (GR)*	GR2	8	44.1666	26.1333	Hap_19	1M,1F	OQ985136
						Hap_3	2M,2F	OQ985080
						Hap_29	1F	OQ985150
						Hap_30	1F	OQ985153
24	Copou (IS)	IS3	1	47.1666	27.6	Hap_3	1F	OQ985081
25	Darabani (BT)	BT2	1	48.20084	26.57484	Hap_3	1F	OQ985082
26	Dragu (SJ)	SJ	3	47.0166	23.4	Hap_3	1M,1F	OQ985083
						Hap_15	1M	OQ985131
27	Dubova (MH)*	MH	1	44.6166	22.2666	Hap_3	1M	OQ985084
28	Frumușeni (AR)	AR1	1	46.11333	21.4776	Hap_1	1M	OQ985058
29	Giroc (TM)*	TM2	10	45.66053	21.26218	Hap_3	6M,1F	OQ985079
						Hap_27	1M	OQ985148
						Hap_33	1F	OQ985156
						Hap_34	1M	OQ985157
30	Gorovei (BT)	BT3	1	47.8833	26.35	Hap_3	1M	OQ985086
31	Gura Teghii (BZ)	BZ3	1	45.4838	26.4217	Hap_3	1M	OQ985087
32	Gușterița (SB)	SB1	1	45.8166	24.1833	Hap_3	1M	OQ985088
33	Măcin (TL)*	TL4	2	45.22083	28.29045	Hap_3	1M	OQ985089
						Hap_29	1M	OQ985152
34	Mediaș (SB)	SB2	1	46.18725	24.32766	Hap_3	1M	OQ985090
35	Miclăușeni (IS)	IS2	2	47.10935	26.93468	Hap_3	2M	OQ985091
36	Movileni (GL)*	GL4	1	45.75	27.3666	Hap_3	1F	OQ985092
37	Odobești (BC)*	BC2	2	46.62905	27.12937	Hap_3	2M	OQ985093
38	Olănești (VL)	VL1	3	45.1857	24.2609	Hap_3	2F	OQ985094
						Hap_35	1F	OQ985158
39	Pardoși (BZ)	BZ4	3	45.47091	26.88486	Hap_3	3F	OQ985095

Table 1. (continued)

№	Population (Locality)	Pop code	N	Latitude	Longitude	Haplotype	Sex	GenBank accession number
40	Păușa (BH)	BH2	7	46.9334	21.8558	Hap_3	3M	OQ985096
						Hap_5	1M	OQ985116
						Hap_6	1M	OQ985118
						Hap_7	1M	OQ985120
						Hap_8	1M	OQ985121
41	Petrești (VN)	VN1	1	45.7251	27.2297	Hap_3	1M	OQ985097
42	Petriș (AR)*	AR2	1	46.0503	22.3788	Hap_2	1M	OQ985059
43	Piatra Neamț (NT)*	NT2	1	46.9166	26.3333	Hap_3	1M	OQ985098
44	Pișchia (TM)	TM1	8	45.9255	21.3810	Hap_3	7M	OQ985085
						Hap_20	1M	OQ985138
45	Piscoiu (GJ)	GJ1	13	44.8221	23.7334	Hap_3	6M,4F	OQ985099
						Hap_13	1M	OQ985128
						Hap_14	1M,1F	OQ985129
46	Râmnicu Sărat (BZ)	BZ5	2	45.3833	27.05	Hap_3	1M,1F	OQ985100
47	Reghin (MS)*	MS	5	46.81161	24.69655	Hap_3	5M	OQ985101
48	Rohia (MM)*	MM2	1	47.2426	23.5115	Hap_3	1F	OQ985102
49	Schulerwald (BN)*	BN2	5	47.1315	24.4644	Hap_3	3M	OQ985103
						Hap_9	1M	OQ985123
						Hap_10	1M	OQ985125
50	Secuieni (BC)*	BC3	2	46.62904	27.12937	Hap_3	1M	OQ985104
						Hap_4	1M	OQ985115
51	Snagov (IF)	IF5	2	44.700	26.1833	Hap_3	2M	OQ985105
52	Supuru (SM)	SM3	2	47.42823	22.71407	Hap_3	2M	OQ985106
53	Șuvara Sașilor (SB)*	SB3	1	45.6705	24.22648	Hap_3	1M	OQ985107
54	Tălășmani (GL)*	GL6	3	46.11586	27.83906	Hap_3	2M	OQ985108
						Hap_22	1M	OQ985140
55	Tecuci (GL)	GL7	1	45.85689	27.42279	Hap_3	1M	OQ985109
56	Tismana (GJ)*	GJ2	2	45.06726	22.94867	Hap_3	1F	OQ985110
						Hap_15	1F	OQ985130
57	Topleț (CS)*	CS	2	44.8	22.4	Hap_3	1M	OQ985111
						Hap_12	1F	OQ985127
58	Topolog (TL)*	TL5	2	44.86645	28.42157	Hap_3	1F	OQ985112
						Hap_32	1M	OQ985155
59	Urechești (VN)	VN2	2	45.6	27.0666	Hap_3	1M	OQ985113
						Hap_36	1M	OQ985159
60	Vaslui (VS)*	VS2	1	46.3833	28.05	Hap_3	1M	OQ985114

average number of individuals per haplotype was 1.42 with 73.4% of all individuals presenting the most common haplotype.

The genetic diversity indices were calculated for 16 populations that counted over three sequenced individuals. The results varied considerably across populations. Populations with high values for genetic diversity (Hd > 0.75) were found in Galați, Bihor and Giurgiu counties in the localities Bălăbănești (GL1), Păușa (BH2) and Comana (GR2), respectively. Moderate values of genetic diversities were found in six populations (Hd > 0.5) and low genetic diversities were found in Bârnova (IS1) and Pardoși (BZ4) populations with null values.

The haplotype network also depicts the distribution of males and females between the identified haplotypes (unknown sex is presented in a separate category). The network, constructed under the median joining algorithm, has a star-like topology, with a central haplotype that contains most of the specimens, and 35 other haplotypes linked to the central one by one or two mutations (Fig. 2). Most individuals (138) share the central haplotype, while the other 35 haplotypes are represented by one to three samples. In our samples, eight haplotypes were shared by females and males, while

Table 2. Genetic diversity indices for the analysed regions based on ND1 sequences. N=number of samples with ND1 sequences; h= number of haplotypes; Hd=haplotype diversity; π= nucleotide diversity; S=number of variable sites; Tajima's D, Fu's Fs, neutrality tests. *=statistically significant.

	Pop	N	h	Hd	π	S	Tajima's D	Fu's Fs
1	**GR1**	25	9	0.54667	0.00109	8	-2.21599*	-8.504*
2	**GJ1**	13	3	0.41026	0.00074	2	-0.90920 (P>0.10)	-0.790 (P=0.224)
7	**GL1**	11	6	0.80000	0.00179	5	-1.46460 (P>0.10)	-3.412*
3	**GL3**	11	2	0.18182	0.00031	1	-1.12850 (P>0.10)	-0.410 (P=0.320)
4	**TM2**	10	4	0.53333	0.00102	3	-1.56222 (P>0.05)	-1.964 (P=0.098)
5	**GR2**	8	4	0.75000	0.00158	3	-0.81246 (P>0.10)	-1.387 (P=0.152)
9	**TM1**	8	2	0.25000	0.00043	1	-1.05482 (P>0.10)	-0.182 (P=0.354)
6	**BH2**	7	5	0.85714	0.00194	4	1.43414 (P>0.10)	-2.858*
8	**IS1**	7	1	0.00000	0.00000	0	-	-
10	**BN2**	5	3	0.70000	0.00136	2	-0.97256 (P>0.10)	-0.829 (P=0.244)
11	**MS**	5	1	0.00000	0.00000	0	-	-
12	**BZ2**	3	2	0.66667	0.00113	1	-	-
13	**BZ4**	3	1	0.00000	0.00000	0	-	-
14	**GL6**	3	2	0.66667	0.00227	2	-	-
15	**SJ**	3	2	0.66667	0.00113	1	-	-
16	**VL1**	3	2	0.66667	0.00227	2	-	-
	Total	188	36	0.461	0.00102	35	-2.63574*	-5.64498*
	Males	140	29	0.44861	0.00099	27	-2.59743*	-50.423
	Females	44	14	0.50846	0.00116	15	-2.51449*	-16.387

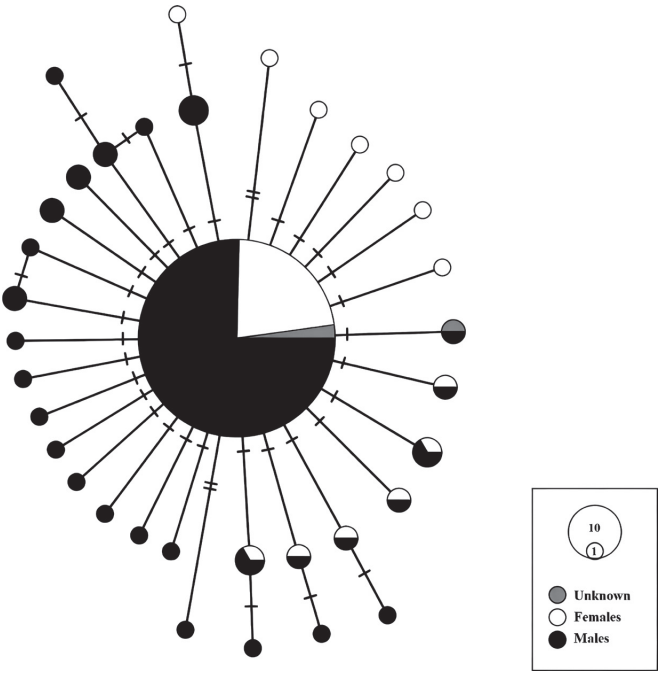

Figure 2. Median Joining network constructed using mitochondrial ND1 sequences of *Lucanus cervus*, showing also the haplotype distribution by sex. Males are represented in black, females in white and individuals of unknown sex in grey. Each haplotype is represented by a circle, circles are proportionate to sample size. Hash marks on lines connecting haplotypes indicate the number of base pair changes between haplotypes.

21 were private for males and seven were private for females. The central haplotype is present in 55 sampling points out of which 27 are present in SACs.

The neutrality tests (Tajima's D; Fu's Fs) had negative values and proved statistically significant ($p < 0.05$). These results are consistent with the scenario of demographic expansion that took place in the recent history of the population (Carneiro de Melo Moura et al. 2019). The mismatch distribution followed an L-shaped outline which is expected for star-like topologies (Fig. 3), indicating the occurrence of a demographic and/or spatial expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992; Lopes et al. 2007).

The IBD analysis performed on individuals, as well as on populations, showed a rather low (although significant) correlation (Rxy = 0.177 and pxy = 0.02).

Discussions

ND1 is a mitochondrial DNA (mtDNA) marker commonly used in molecular biology and evolutionary genetics of insects (Kosovac et al. 2018; Zhao et al. 2022). ND1 is a

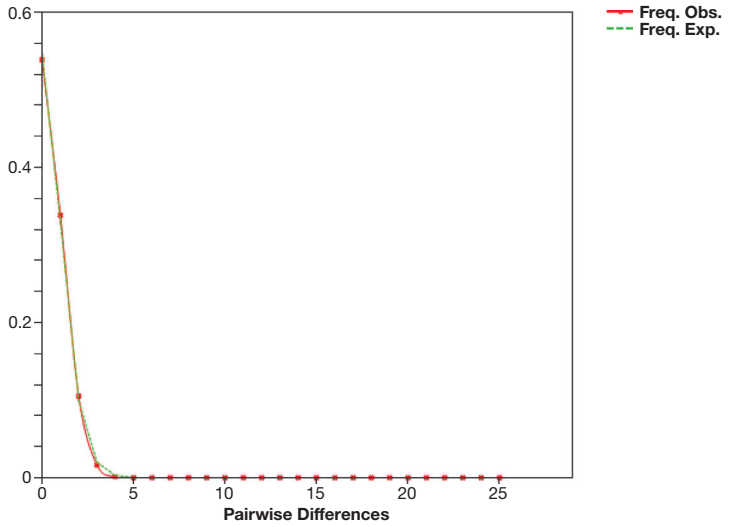

Figure 3. Mismatch distribution of *Lucanus cervus* ND1 sequences. On the x-axes we represented the number of nucleotide differences.

useful tool used in mtDNA-based population genetics studies to investigate genetic diversity and structure among populations, as well as to infer historical demographic events such as population expansions or contractions. It is also frequently used in phylogenetic studies to reconstruct evolutionary relationships among different species or populations (Wendt et al. 2021).

We obtained a moderate diversity of haplotypes in our analyzed samples (Hd = 0.461), with 36 haplotypes identified in 188 samples. The obtained network model suggests a recent and rapid expansion of the population from a single ancestor. This can occur, for example, due to a founder effect, when a small group of individuals establish a new population with limited genetic variation. Also, the star-like pattern represented by the association of one common haplotype with others in lower frequencies or private haplotypes is frequently attributed to populations that have undergone recent range expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992; Lopes et al. 2007). However, it's important to note that the star shape model with a central haplotype shared by many samples is a simplification of the complex patterns of genetic diversity that exist in real populations. In reality, populations can have multiple founders and can experience genetic drift, gene flow, and natural selection, all of which can affect the distribution and accumulation of genetic mutations. This result is also confirmed by the mismatch distribution analysis and the Tajima and Fu neutrality tests. Our results are similar to the ones found by Cox et al. (2019) after analyzing COI sequences of stag beetle samples collected from all over Europe. They found the same star-like distribution of haplotypes in all sampled populations apart from Greece, where the haplotypes were much diversified and displayed a more dendritical structure (Cox et al. 2019).

Our analyses on male *versus* female samples yielded similar results (Fig. 2). Our sample is biased towards males (with 140 males and only 44 females), and both the proportion of individuals in the central haplotype and the distribution of private haplotypes in males and females is not significantly different ($p < 0.05$). The presence of private haplotypes for male and female populations is most likely due to the small sample size. The bias towards males in samples is well known in monitoring strategies (Katušić et al. 2017; Bardiani et al. 2017) and is explainable at least in part by the fact that females are more difficult to observe, as they lay their eggs in the ground at the base of trees, and at the last deposition they die inthe ground, the remains of the body not being accessible (Tini et al. 2017a,b). Yet another possible explanation for the observed sex bias could be the fact that a larger number of males are necessary in order to preserve an optimal effective population size. The larger male population would cope with potential stronger selection pressure on males, given that they are much more visible to animals praying on them, for example. However, our results (no significant difference between males in females concerning the mtDNA haplotype distribution) did not support such a hypothesis.

The IBD analysis involves comparing the genetic distance between individuals or populations with their geographic distance. If there is a positive correlation between genetic and geographic distance, this suggests that gene flow is limited between populations that are far apart, and genetic drift plays an important role in the distribution of genetic diversity. Conversely, if there is no correlation between genetic and geographic distance, this indicates that gene flow is high, and genetic diversity is shaped primarily by gene flow rather than genetic drift. Our IBD analysis revealed a weak correlation between genetic and geographic distances which would imply that there is a limited gene flow between populations that are far apart and that the genetic drift may play a more significant role in shaping the genetic diversity of the populations. However, given the reduced sample size of many populations in our study, the issue of IBD needs further exploration with appropriate sample sizes.

The population genetics indices revealed various degrees of diversity in the 16 analyzed populations. Many of the populations with low genetic diversity were found in SACs from the Natura 2000 Network, while populations that are not included in protected areas (e.g., Păușa and Bălăbănești) proved to have higher values of the calculated genetic diversity indices. This underlines the need to review the designated protection sites for this species. One needs to keep in mind that the small sample size of our populations might also be responsible for the observed results. Yet another reason for the reduced variability observed in some cases might be the evolutionary rate of the ND1 marker itself. Although it has been used in many previous population genetic studies of insects, ND1 may be too slow-evolving to be informative to assess population structure associated with habitat fragmentation. Fragmentation of woodland habitats, one of the preferred habitats of *L. cervus*, is accelerated in recent years (Munteanu et al. 2016; Öder et al. 2021; Tudoran et al. 2021). Therefore, faster evolving markers may have the potential to resolve population structure related to recent events/pressures and could provide a more reliable image of the genetic

diversity of the investigated population. This, in turn, could suggest more adequate conservation measures to be taken for populations in decline.

Situated at the intersection of Eastern, Central, Southeastern Europe and the Black Sea, Romania is considered one of the European "biodiversity hotspots". Ecologically, the country is characterized by a diverse range of ecosystems, as it encompasses parts of five major vegetation ecoregions, namely the Carpathian Montane Coniferous Forests, Pannonian Mixed Forests, Central European Mixed Forests, East European Forest Steppe and Pontic Steppe (Gabrielsen and Bosch 2003). In Romania, *L. cervus* is present in five biogeographical regions: Alpine (15 Natura 2000 designated sites), Continental (65 sites), Pannonian (6 sites), Black Sea (1 site) and Steppic (8 sites). The status of the species was assessed as favorable in the national report of Romania, for the period 2013–2018, for all five regions (https://eunis.eea.europa.eu). In our study, a little over half of the collecting points falls outside Natura 2000 sites, and only 28 of the sampled populations are distributed in protected areas. Those not included are situated in urban areas or are found in woodland areas, on the outskirts of localities, that do not bear a protection status. The situation is correlated with the fact that this species is well known to have urban areas as preferred habitats, beside forest habitats (Bardiani et al. 2017). Therefore, the conservation of the species in Romania should also take into consideration its presence in parks and other urban areas that are not under protective legislation.

The examination of genetic diversity is essential in the case of species like *L. cervus*, which have a protracted life cycle and inhabit fragmented habitats, to be able to implement efficient conservation measures. In order to gain a more comprehensive understanding of the genetic makeup *of L. cervus*, it would be beneficial to employ both mitochondrial and nuclear markers in the analysis, given the distinct evolutionary pressures that act on each type of marker.

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