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SHORT COMMUNICATION

First genetic analysis of introduced axis deer from Croatia

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Abstract

The axis deer (*Axis axis*) is a species of ungulate native to the Indian subcontinent. In the nineteenth and twentieth centuries, the axis deer was introduced to many regions of the world, where it established non-native free-ranging populations. The introduction of the axis deer to Croatia resulted in three populations that still live on the Adriatic islands.

In this study, two new mitochondrial DNA control region (D-loop) haplotypes were identified in 39 axis deer samples from two Adriatic islands Rab and Dugi Otok in Croatia. Two distinct D-loop haplotypes found in Croatian axis deer populations indicate that axis deer in Croatia were introduced from at least two maternal lineages. Genetic differentiation between populations was quite low and not significant. Haplotype (0.497) and nucleotide (0.006) diversity of Croatian axis deer was similar to that of axis deer from Queensland, Australia (0.461 and 0.002, respectively). For a better understanding of the origin and genetic diversity of the introduced axis deer from Croatia, analysis of native populations and the addition of highly variable nuclear markers is required.

Keywords *Axis axis* · Cervidae · Chital · D-loop · mtDNA · Non-native

The Cervidae is a group of antler-bearing ungulates of varying size with a complex taxonomy of about 53 species and 169 extant taxa with a worldwide distribution (Mattioli 2011). Ungulates are the most widespread and numerous large terrestrial mammals in Europe with about 20 species. These species play an important role in the ecosystem, which is even more pronounced in the case of non-native species that pose a threat to native species and/or habitats. Among all the introduced non-native groups of mammal species in Europe, the ungulates stand out as one of the most important game groups in all European countries (Carpio et al. 2017). The most common ungulate species in Europe such as red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) have been the subject to numerous scientific studies (Linnell and Zachos 2011). On the other hand, some non-native species are already the subject of scientific studies (e.g., sika deer—*Cervus nippon*, Linnell and Zachos 2011), while others are poorly studied (e.g., axis deer—*Axis axis*, Šprem and Zachos 2020).

Axis deer or chital originate from the Indian subcontinent, where population size has declined rapidly due to recent human activities such as uncontrolled hunting or habitat fragmentation due to urbanisation, and agricultural practise (Abbas et al. 2016), but the species is still classified as 'Least Concern' by the IUCN (International Union for Conservation of Nature) (Duckworth et al. 2015). In the nineteenth and twentieth centuries, axis deer were introduced to many regions of the world, where they established non-native free-ranging populations (Šprem and Zachos 2020). There have been several attempts at the introduction of axis deer individuals by various European countries, but it has been successful only in Croatia, where three populations still live on the Adriatic islands. The genetic origin of the introduced individuals is not known, but it is suspected that they were imported by the German wildlife trader Carl Hagenbeck (Šprem et al. 2008; Kusak and Krapinec 2010). The first introduction occurred in 1911 at Brijuni Islands, as

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it was an acclimatisation station for wild animals from the tropical belt that were on their way to European zoos (Šprem et al. 2008; Kusak and Krapinec 2010). In 1974, eight individuals (two males and six females) were translocated from Brijuni Islands to the Kalifront Peninsula part of the island of Rab and have since been kept for hunting purposes (Centore et al. 2018). Another population established on the island of Dugi Otok dates back to 2012, when 13 individuals originating from Brijuni Islands escaped from a fenced area. There were also several other attempts to translocate axis deer in different parts of Croatia, but they all failed due to the poor adaptation of this species to the continental climate (Krapinec 2001). Therefore, the axis deer from the Adriatic Islands in Croatia are the only free-living European populations (for more details see Šprem and Zachos 2020).

Axis deer have rarely been the subject of genetic studies worldwide, and there is still a lack of genetic data for European (Croatian) axis deer. In a recent methodological paper, Paul et al. (2019) describe a set of molecular markers to identify species and sex of five cervids (including axis deer) in northern India. A few studies confirmed hybridization between axis deer and some other cervid species, and hybridization with sika deer was detected in a deer farm in the USA (Asher et al. 1999). Hybridization between axis deer and hog deer (*Hyelaphus porcinus*) has been reported twice, the first in a check-list of mammalian hybrids by Gray (1954) and the second, more recent in Australia by Hill et al. (2019). In addition, Gray (1954) reported also axis deer hybridization with red deer and white-tailed deer (*Odocoileus virginianus*). According to Iacolina et al. (2019) no hybridization with axis deer has been reported in Europe. The mitochondrial DNA (mtDNA) variation of Pakistani axis deer populations was investigated in the study by Abbas et al. (2016), where they found low mitochondrial genetic differentiation between the studied populations.

In this study, we present a first assessment of the maternal genetic variability of the axis deer populations in Croatia. Since mtDNA sequences represent a useful tool for assessing genetic diversity and variability between populations (Tešija and Safner 2021), the aims of this study were the following: (i) to determine the genetic diversity of the mtDNA control region in axis deer populations from the Adriatic islands of Rab and Dugi Otok in Croatia; (ii) to combine the haplotypes found in the studied populations with those previously identified and available in GenBank, to improve our understanding of the geographic variation of axis deer.

The study was conducted on the islands of Rab and Dugi Otok in Croatia. Rab Island is located in the northeastern Adriatic Sea (44°47′24″N, 14°40′10″E). The area is a forest ecosystem consisting of carbonate rocks and predominantly holm oak (*Quercus ilex*) and manna ash (*Fraxinus ornus*) (Ugarković and Ugarković 2013; Kavčić et al. 2019), at altitudes between sea level and 94 m. Dugi Otok Island at altitudes up to 338 m a.s.l. is located in the central Adriatic Sea (43°57′56″N, 15°06′24″E). In the northern part scrub and the degradation stage of holm oak forest (*Quercus ilex*) with myrtle (*Myrtus communis*) predominate, while in the southern part of the island Aleppo pine forests (*Pinus halepensis*) and holm oaks occur (Vukelić 2012). According to the Köppen climate classification, Rab Island belongs to the moderately humid climate with hot summers, climate type Cfa, while Dugi Otok Island belongs to the Mediterranean climate with hot summers, climate type Csa (Šegota and Filipčić 2003). Population size is estimated by visual counts conducted by gamekeepers during field observations twice a year (in spring and autumn); on the island of Rab there are 78 individuals (Centore et al. 2018) and on Dugi Otok 46 individuals (Šprem N 2020, pers. obs.).

Tissue samples were collected from 42 axis deer (Rab Island, $n = 35$; Dugi Otok Island, $n = 7$) during regular culls between 2017 and 2021 in accordance with wildlife management plans.

DNA was extracted from tissue samples, using the Tissue DNA Mini Kit – S line (VWR International, Leuven, Belgium; thereafter: PeqLab), according to the manufacturer's instructions. DNA fragments of the mitochondrial control region (D-loop) were amplified according to the protocol and using the primers described in Hill et al. (2019) by ready to use Kapa2G Fast multiplex mix (Kapa Biosystems) in a 20-μL reaction containing 3.0 mM $MgCl_2$ and 0.5 mM of forward and reverse primer. Cycling conditions consisted of an initial stage of 95 °C for 3 min followed by 35 cycles of denaturation (30 s at 95 °C), primer annealing (30 s at 58 °C), extension (40 s at 72 °C) and 10 min of final extension at 72 °C.

Sequencing reactions were carried out on both DNA strands. Ambiguous results were checked by multiple sequencing of a new DNA extraction from the same individual.

Sequencing was performed on SeqStudio sequencer (Thermofischer Scientific) using BigDye Terminator chemistry (Applied Biosystems, Foster City, CA, USA).

The Codoncode Aligner program v 3. 7. 1 (CodonCodes Inc., Ewing et al. 1998) was used to align forward and reverse sequences. The resulting 576 bp consensus sequence was aligned using CLUSTALW (version 4.0, Thompson et al. 1997) implemented in the MEGA package (version X, Kumar et al. 2018). As pseudogenes are known to represent a serious source of error for mitochondrial phylogenies (Triant and DeWoody 2009), control region sequences were checked for the absence of stop codons and indels.

Genetic diversity indices (number of haplotypes, polymorphic sites, haplotype and nucleotide diversities, mean number of pairwise differences) were calculated using Arlequin 3.5 (Excoffier and Lischer 2010) for the whole dataset and individual populations. The Tamura 3-parameter model was used to correct for the number of nucleotide substitutons per site, since it was the best nucleotide model proposed by MEGA. Differentiation between the two populations was estimated with pairwise F_{ST} using Arlequin with 1000 permutations.

To evaluate the phylogenetic position of Croatian axis deer, additional mtDNA control region (D-loop) haplotypes of axis deer were downloaded from GenBank and included in our dataset (JN632599: Hassanin et al. 2012; MN226865-MN226866: Hill et al. 2019; JN596132-JN596143, JN596145-JN596148, JN596150-JN596151, JN596153-JN596156: Kumar et al. unpublished, direct submission to GenBank). The geographic origin of these sequences were India and northern Queensland in Australia. The GenBank sequences were shorter but there was an overlap with ours in 320 bp, and the final alignment comprised 25 sequences and 16 haplotypes. To visualize the phylogenetic relationships among haplotypes, a median-joining haplotype network was created using the program PopART (Leigh and Bryant 2015). Network analyses are well suited to assess relationships among haplotypes in intraspecific studies because in contrast to phylogenetic trees, they take into account the presence of ancestral haplotypes and likely evolutionary pathways (Zachos 2009).

Three samples out of 42 were not successfully amplified due to highly degraded DNA. Therefore, the following analyses were conducted on 39 mtDNA control region (D-loop) sequences from the two Croatian axis deer populations. A 576-bp D-loop fragment amplified in this study corresponds to positions 15,460–16,035 of the complete axis deer mitochondrial genome (NC_020680: Hassanin et al. 2012). A total of seven polymorphic sites were identified (7 transitions), resulting in two (and previously unknown) haplotypes with a sequence divergence of 1.22% (GenBank Accession numbers MZ421332 and MZ421333). The presence of two maternal lineages indicates that axis deer in Croatia were established by females going back to at least two different maternal lineages.

The total haplotype (Hd) and nucleotide diversities (π) were 0.497 and 0.006, respectively, and the average number of nucleotide differences (k) was 3.522 (Table 1). Haplotype diversity within the population of Dugi Otok was 0.286 and for Rab 0.514, while nucleotide diversity was 0.004 for Dugi Otok and 0.006 for Rab. In the population of Dugi Otok, all but one of the seven analysed individuals carried the same haplotype.

Genetic differentiation was quite low and not significant between sampled populations ($F_{ST} = 0.119$, $P > 0.204$). The F_{ST} value means 88.1% variance at the intrapopulation level.

The structure of the median-joining haplotype network created by combining our data and GenBank sequences from India and Australia showed the low number of mutations between haplotypes (mean 5.5), indicating either that they originated from the same evolutionary unit or that mitochondrial divergence in the species is overall low (Fig. 1). Two different haplotypes are present in both populations studied and could also reflect the two past introduction events of axis deer in Croatia. From the literature review the first introduction to Brijuni Island occurred in 1911 while the second event occurred in the 1960s to 1970s, but with animals of unknown origin (Bojović 1987; Šprem et al. 2008). The haplotypes detected in the Croatian samples were not observed in the native axis deer population from India or in the introduced Australian one (Hill et al. 2019). The Australian population was established by imported individuals from India (Jesser 2005). It should be emphasized that the study by Hill et al. (2019) was primarily on hog deer and the number of axis deer samples analyzed (35) could at best provide preliminary results, considering that the population size of axis deer in Australia has been estimated at 44,000 individuals (Kelly et al. 2021). However, both Croatian haplotypes were separated from their nearest neighbor by a maximum of three mutational steps (Fig. 1).

Table 1 Genetic diversity indices based on a 576-bp fragment of D-loop for axis deer (*Axis axis*) in Croatia

Island/population	Rab	Dugi Otok	All samples
No. samples	32	7	39
No. haplotypes	2	2	2
No. polymorphic sites (S)	7	7	7
Haplotype diversity (Hd)	0.514	0.286	0.497
Nucleotide diversity (π)	0.006	0.004	0.006
Mean no. of pairwise differences (k)	3.646	2.026	3.522

The present study showed a low level of haplotype diversity in axis deer from the Adriatic islands of Rab and Dugi Otok in Croatia, in line with a founder effect when they were introduced. This is comparable to two D-loop haplotypes found in 35 individuals of axis deer from northern Queensland, Australia (Hill et al. 2019). Similarly, haplotype (0.497) and nucleotide (0.006) diversity of the Croatian axis deer match the values found in the Australian axis deer sample (0.461 and 0.002, respectively). In contrast, 25 D-loop haplotypes are reported in GenBank for axis deer from their natural range in India.

A similarly low number of haplotypes has been reported for other ungulate species recently introduced to Europe. D-loop analysis of 26 individuals of sika deer (*Cervus nippon*) from two regions in continental Europe, Kadyny Forest (Poland) and Kaliningrad District (Russia), revealed only two haplotypes, with each population monomorphic for one of them (Biedrzycka et al. 2012). A slightly higher number of mtDNA control region haplotypes (in total four) were found in European populations of Barbary sheep, or aoudad

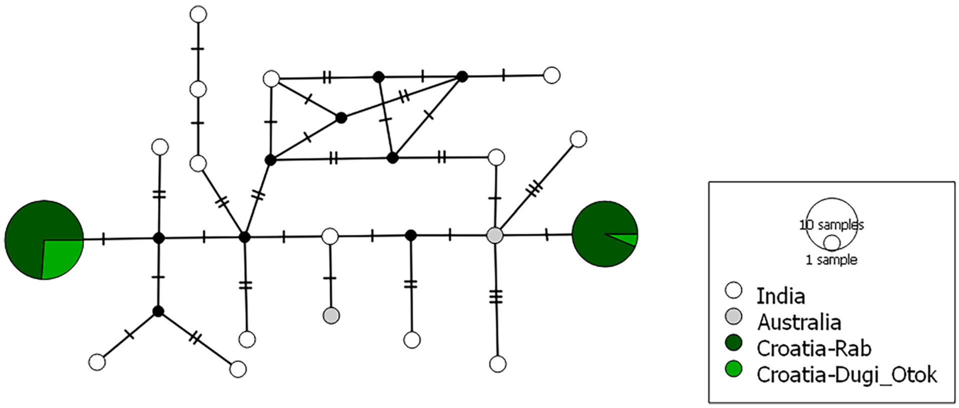

Fig. 1 Median-joining network based on a 320-bp fragment of the mitochondrial DNA control region (D-loop) showing the relationships of Croatian axis deer from the islands of Rab and Dugi Otok and other populations. Haplotypes for the two Croatian populations are represented by circles whose size is proportional to the number of individuals. The number of mutations separating the nodes is represented by dashes

(*Ammotragus lervia*). Sierra Espuña in Spain was the only population where more than one haplotype was detected for this species (Stipoljev el al. 2021).

Previous studies on mtDNA diversity in European fallow deer (*Dama dama dama*) have generally shown low haplotype diversity in most populations (Baker et al. 2017; Kusza et al. 2018). The haplotype diversity found in our study is lower than the Hd values reported for the majority of European fallow deer populations, ranging from 0 to 0.962 (mean 0.664), while the low nucleotide diversity value is similar to the reported π values, ranging from 0 to 0.0134 (mean 0.006) (Kusza et al. 2018).

On the other hand, the native red deer (*Cervus elaphus*), which is one of the most widespread European ungulate species, has generally high D-loop haplotype diversity across Europe, with an overall Hd value of 0.96 (Skog et al. 2009).

Our study showed that Croatian axis deer have similar mitochondrial genetic diversity indices as found in other recently introduced populations. Genetic analysis of the native population and the addition of highly variable nuclear markers (e.g., microsatellites or genome-wide SNPs) are necessary for a better understanding of the origin and genetic diversity of axis deer populations in Croatia.

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Authors' contributions NŠ and SS wrote all drafts of the manuscript. NŠ and EB conceptualized the framework and revised all drafts of the manuscript. SS did the statistical analyses. DU obtained and arranged raw data.

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Availability of data and materials All the data supporting the results in the manuscript are included in the Supplementary material and are available in the GenBank repository (accession numbers MZ421332—MZ421333).

Code availability Not applicable.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Consent for publication Not applicable.

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