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Original Article

How we look: European wild mouflon and feral domestic sheep hybrids

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Abstract

Hybridization between wild animals and feral individuals from closely related domestic species can occur when they share the same habitat. Such events are generally regarded as a threat to the genetic integrity and survival of established wild populations. The aim of this study is to confirm a hybridization between European mouflon and feral sheep on the Adriatic island Dugi Otok, where a group of individuals phenotypically indicative of a possible introgression was photographed. Using an ovine medium-density SNP array genotypes, we provided genetic evidence of recent gene admixture between the two species. The ADMIXTURE analysis suggests that two hybrid individuals had 82.2% and 94.1% of their ancestry attributable to feral sheep, respectively, with the rest of the mouflon in origin. NEWHYBRIDS analysis estimated the combined probabilities for the putative hybrid individuals belonging to one of the hybrid classes at 87.0% and 90.1%, respectively. The main phenotypic differences in the hybrids were observed in the shape of the horns, a stronger horn circumference than in the mouflon and weaker than for the sheep, pronounced horn rings, a shorter tail, and intermediate color. We therefore recommend constant monitoring of the purity of the mouflon population to maintain its genetic integrity. In addition, all feral sheep should be removed from the wild, and released sheep raised in semi-wild conditions should be better controlled.

Key words: crossbreeding, gene introgression, livestock, *Ovis gmelinimusimon*, SNPs.

Hybridization, the reproduction of individuals from genetically distinguishable populations, affects about 10% of animal species (Mallet 2005) and has significant implications for species evolution, conservation, and management (Adavoudi and Pilot 2022). It can occur naturally, due to secondary contact by range expansion between previously isolated populations (Allendorf et al. 2013), or can be caused by human activities, such as the (un)controlled (re)introduction of exotic and domesticated species (Iacolina et al. 2019) which can cause hybridization between different wildlife (sub)species or livestock. Hybridization is often considered a threat to the genetic integrity and survival of populations through genetic swamping and outbreeding depression but can also have positive consequences, such as the introduction of new adaptive variation and increased fitness performance (Adavoudi and Pilot 2022).

Hybridization can be detected in several ways, either through phenotypic traits (Moroni et al. 2022) or through the use of various molecular genetic data (Adavoudi and Pilot 2022). Confirmation of hybridization based just on phenotypic traits is difficult, especially when multiple backcrosses have occurred (Somenzi et al. 2020). The most common molecular genetic markers used to detect hybridization are mtDNA, Y chromosome, autosomal microsatellites, and more recently single-nucleotide polymorphisms (SNPs) (Iacolina et al. 2019). SNPs can be genotyped in large numbers (hundreds of thousands to millions) using arrays or next-generation sequencing, making them useful for identifying weak hybridization signals (Grossen et al. 2014; Iacolina et al. 2018; Somenzi et al. 2020).

The European mouflon *Ovis gmelini musimon* (Figure 1A.) was introduced by humans to the Mediterranean islands of Corsica and Sardinia during the first wave of domestication about 6,000-7,000 years ago, as supported by archaeological records in Neolithic sites (Vigne 1992). Since the 18th century, the mouflon has been introduced as a game animal in many European countries, and the first introduction in Croatia occurred in 1900, using animals of uncertain origin (Ferenčaković et al. 2013). Over time, the mouflon became an important game species and has spread throughout the country, especially in the Mediterranean part and on the Adriatic islands (Kusak and Krapinec 2010), where it frequently comes into contact with livestock, predominantly domestic sheep. On some Mediterranean islands, specifically ones with

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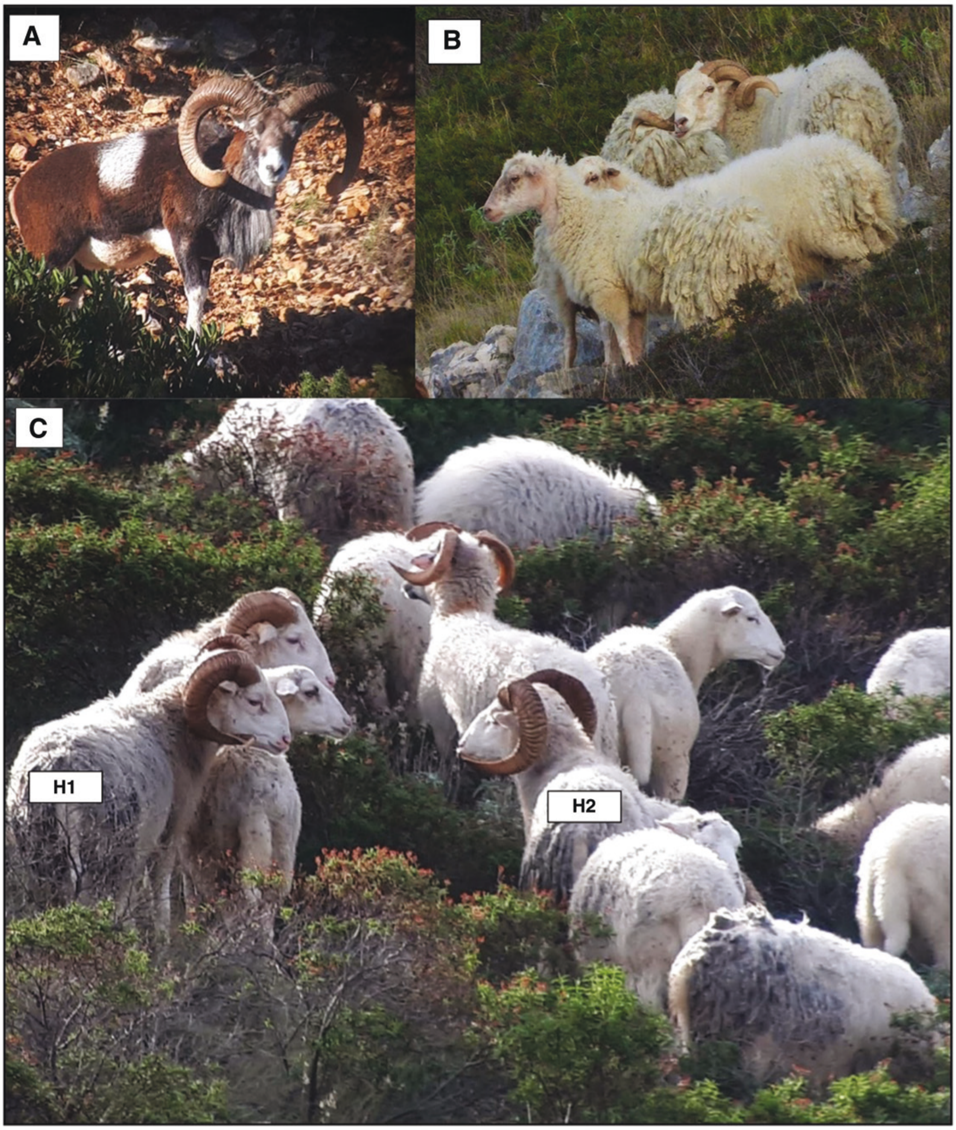

Figure 1. (A) Photo of European mouflon; (B) photo of feral sheep; (C) photo evidence of the group of hybrids, in the foreground are two rams aged three years with individual acronyms (H1 and H2), that are genotyped in this study and confirmed as *Ovis gmelini musimon* × *Ovis sp.* hybrids. Photo was taken on the island of Dugi Otok (Croatia). Photo copyright: Milutin Žampera.

a long history of traditional sheep rearing, there can be found populations of feral sheep (Figure 1B). Those are composed mostly of the offspring of sheep once kept as domestic, but that were left or escape in the wild, and are now considered feral. On the island of Dugi Otok in 2021, a small group of roughly twenty animals that were phenotypically and behaviorally different from both mouflons and sheep were spotted. Because there are a considerable number of domestic and feral sheep on the island, that share habitat with mouflon, after careful observation, it was determined that this particular group of animals might be hybrids (Figure 1C).

Due to the similarities in their genomes, hybridization between European mouflon and domestic sheep is difficult to detect by using a single locus approach (Lorenzini et al. 2011; Göttert and Perry 2023). SNPs are considered the appropriate genetic tool to resolve the hybridization issues (Ciani et al. 2014; Barbato et al. 2017). Therefore, the aims of this study were to confirm hybrid ancestry of observed phenotypically distinct animals using a 50K SNP chip and to investigate the pattern of introgression between the coexisting population of European mouflon and feral sheep in Adriatic island Dugi Otok.

Materials and Methods

Study area and sample collections

The study was conducted on the island of Dugi Otok in Croatia, located in the central Adriatic Sea (43°57′56 ″N, 15°06′24 ″E). In the northern part, scrub and degradation stage of holm oak forests Quercus ilex with myrtle Myrtus communis predominate, while in the southern part of the island Aleppo pine forests Pinus halepensis with holm oaks occur (Vukelić 2012). According to the Köppen climate classification, the island of Dugi Otok belongs to the Mediterranean climate type Csa (Šegota and Filipčić 2003). Wild ungulates present in the study area are the European mouflon, the axis deer Axis axis, and wild boar Sus scrofa. The study area is also inhabited by feral sheep and feral goats. Tissue samples were collected from 21 mouflon, 13 feral sheep, and 2 assumed hybrids (based on their phenotypic traits; see Figure 1C) during regular culls between 2021 and 2022 in accordance with wildlife management plans (Supplementary Table S1).

DNA extraction and genotyping

DNA was extracted from tissue samples, using the Qiagen QIAamp® DNA kit, following the manufacturer's instructions (Qiagen, Germany). DNA concentration was estimated using a Quibit 3.0, with Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, USA). Samples were genotyped using the commercially available Illumina OvineSNP50 Beadchip. Genotyping on the Illumina platform was undertaken by a single commercial company (Weatherby's, Ireland). Genotype calling was conducted using GenomeStudio Genotyping Module v1.0 (Illumina, San Diego, CA, USA). The manifest and cluster file were provided by Illumina. The quality control and data pruning procedure was carried out using PLINK 1.9 (Purcell et al. 2007). In the pruning procedure the SNPs that were unmapped, mapped to the X and Y chromosomes or on mitochondrial DNA were removed, and only the SNPs located on autosomes were kept for further analysis. Finally, SNPs with call rate (CR) ≤ 95%, minor allele frequency (MAF) ≤ 0.05, and SNPs with observed correlation coefficient (R^2) > 0.1 with any other SNP within the moving window of the size 50 were excluded.

Genetic diversity and population structure

Maximum likelihood analysis of population structure was conducted using ADMIXTURE v1.3 (Alexander and Lange 2011). Clustering solutions for the whole dataset were calculated using supervised analysis for *K* value of 2. All the reference mouflon and feral sheep were designated as the reference samples and two supposed hybrids as individuals with unknown ancestry. The model was run for 10 times, and the average admixture values were calculated for each individual.

Hybrid detection

To estimate posterior probabilities of each individual's assignment as a pure parent (mouflon or feral sheep), first- or second-generation hybrid (F1 or F2), or an F1 backcross with each of the parents, NEWHYBRIDS v.1.1 (Anderson and Thompson 2002) was used, which applies a Gibbs sampler and Markov Chain Monte Carlo method. To further reduce the number of unlinked SNP loci for this analysis, 300 of the most informative SNPs were selected based on their F_{ST} values between parental populations. This was done using R package "hierfstat" (Goudet 2005). NEWHYBRIDS analysis was run using Jeffreys prior probabilities and default genotype proportions for each of the predefined classes. The Markov chain was run for 200,000 sweeps following a burn-in of 50,000

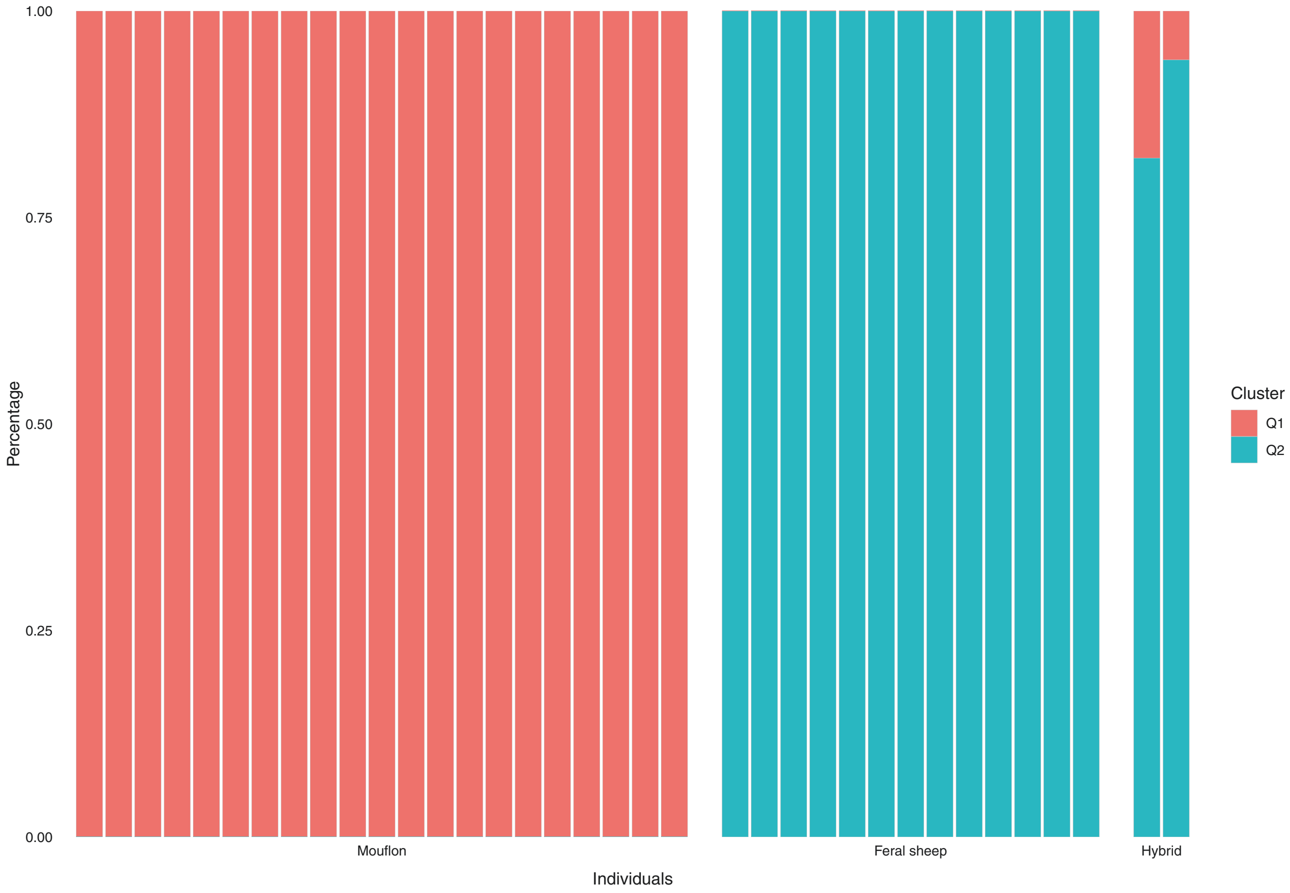

Figure 2. Results of the supervised ADMIXTURE analysis. Each colored bar represents one analyzed individual. Individuals are grouped into the categories of origin (European mouflon, feral sheep and assumed hybrids). Colors represent the average percentage of the genome assigned to the one of the two genetic clusters from 10 runs of the analysis.

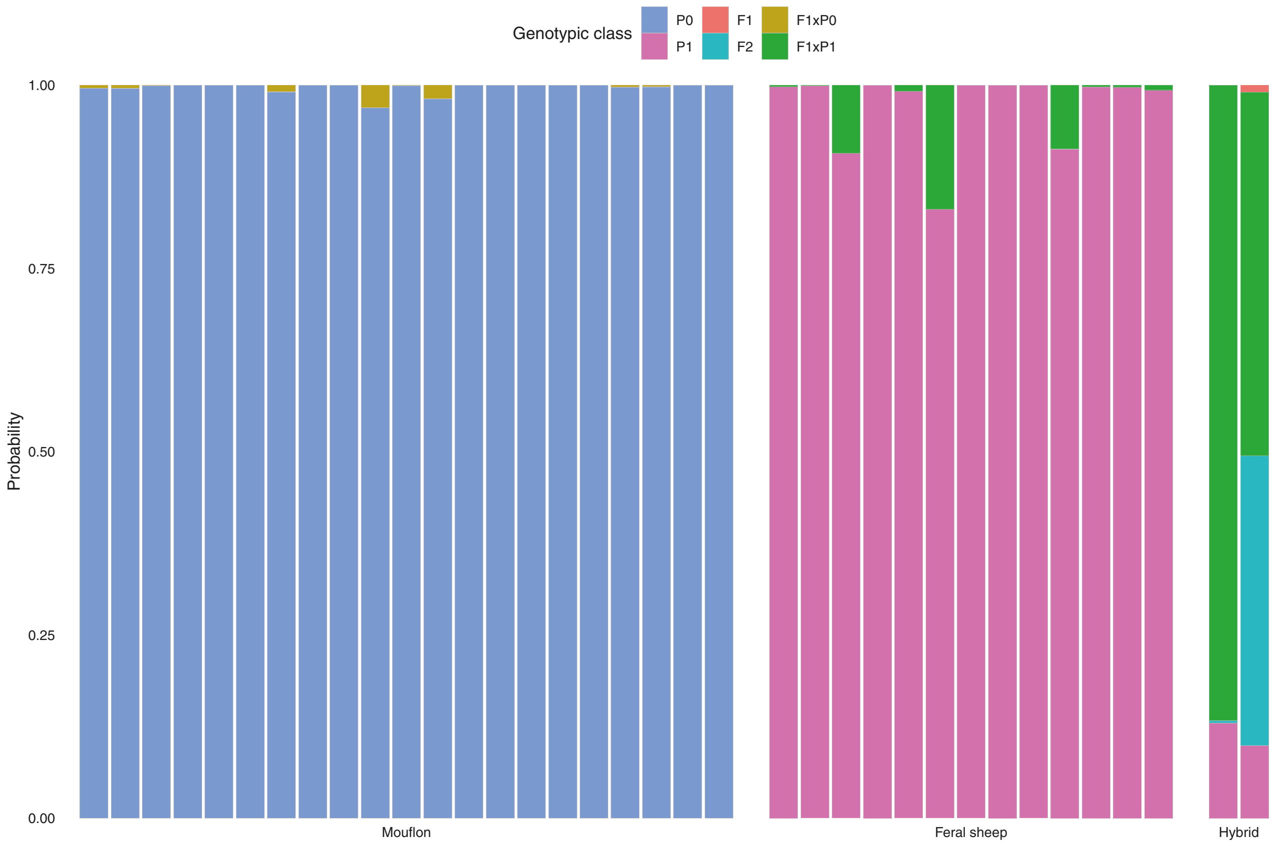

Figure 3. Results of the NEWHYBRID analysis. Each colored bar represents one analyzed individual. Individuals are grouped into the categories of origin (European mouflon, feral sheep and assumed hybrids). Colors represent the probability for each individual to belong to one of predefined genotypic classes (pure European mouflon, pure feral sheep, F1 hybrid, F2 hybrid, backcross with European mouflon, backcross with feral sheep).

iterations. The results of ADMIXTURE and NEWHYBRIDS analyses were visualized using ggplot2 R package (Wickham 2016).

Results

The main phenotypic differences in rams assumed as hybrids were observed in the shape of the horns, a stronger horn circumference than in the mouflon and weaker than for the sheep, an intermediate tail length, and coat color (Figure 1C).

After pruning amplified SNPs based on CR, MAF, and correlation, the final dataset consisted of 36 individuals and 2901 SNP loci.

Results of supervised ADMIXTURE analysis at $K = 2$ clustered the reference samples into genetically distinct mouflon and feral sheep groups (Figure 2), inferred to have over 99.9% of the ancestry matching their phenotype. Assumed hybrid individuals, on the other hand, had 82.2% (individual acronym—H1) and 94.1% (individual acronym—H2) of the genome of the higher reference (feral sheep in both cases).

Results of the NEWHYBRIDS analysis confirmed the assumption that two phenotypically distinct animals were, indeed, hybrids between mouflon and feral sheep. For both individuals, the highest probability (H1—86.7%, and H2—49.6%) is that they are backcross hybrids between F1 generation mouflon × feral sheep and pure feral sheep parents (Figure 3). For the same individuals, the combined probabilities of belonging to one of the hybrid classes were 87.0% and 90.1% respectively. All reference mouflon individuals had probabilities of belonging to the pure mouflon genotypic class higher than 96.0%. Among reference feral sheep individuals, the probabilities of belonging to the pure genotypic class were lower than among mouflon, with the lowest one being 83.0% (individual acronym FS6).

Discussion

In the individuals that were designated as assumed hybrids based on the phenotypic traits, the horn rings were much more pronounced than in domestic/feral sheep, as well as the depth and curvature of the horns. This shape of the horns is typical of mouflon and unlike domestic/feral rams (Figure 1A,B), which are similar in shape to Marco Polo sheep *Ovis ammon polii*. The rings of the horns are also much more pronounced than in domestic/feral sheep, as well as the depth and curvature of the horns (Figure 1C.). Such changes in the shape of the horns are known in mouflon hybrids and have been described much earlier than in this study. They usually have horns of greater spread, length, and basal circumference (Uloth 1972). Other characteristics of hybrids that differ from the original species have also been recognized, such as tail length, coat, woolly fleece, and color (Schröder et al. 2016). In this article, we provide the first photograph of hybrid mouflon × feral sheep individuals, which clearly shows described hybrid phenotypic characteristics. Similar phenotypic changes due to introgression were illustrated by Moroni et al. (2022) in their study of hybrids of Alpine ibex *Capra ibex* and goats, showing a photograph that clearly shows phenotypic changes, such as horn size and shape, body outline size, and coat color.

While ADMIXTURE analysis results show higher levels of admixture in the assumed hybrid individuals than in any of the individuals from both reference populations, it is from the results of the NEWHYBRIDS analysis confirmed that these two individuals are indeed the hybrids between mouflon and feral sheep and that the hybridization events occurred multiple times in past few generations. Based on the high probability (86.7%) that at least one of the assumed hybrids belongs to the backcross between F1 hybrid and feral sheep, it can be assumed that mating between mouflon and feral sheep occurred at least twice in the past two generations. These further prove that the hybrid individuals can survive, successfully reproduce, and have fertile offspring. Observed levels of admixture were much higher than it was estimated in mouflon populations in Corsica and Sardinia (Barbato et al. 2017). Interactions between mouflon and domestic sheep are not uncommon and have occurred frequently since the second wave of domestication, and hybrids between them have been confirmed in several cases (Ciani et al. 2014; Schröder et al. 2016; Barbato et al. 2017). For example, Barbato et al. (2017) recently reported introgressions of both mouflon genome into feral sheep, but also vice versa. They reported that signals of introgression of domestic sheep into mouflon were the strongest in fenced mouflon populations in Sardinia, whereas hybridization signatures were weaker in mainland Europe. Our results gave evidence only to one-way introgression (from mouflon into feral sheep), but this can be the consequence of random sampling (e.g., sample not including mouflon with hybrid ancestry). One additional possible consequence of our sampling design could be the underestimation of detected hybridization events due to the lack of external samples, such as domestic sheep or mouflon from other populations. Due to this, we were not able to estimate the levels of possible hybridization that could have occurred within our reference samples prior to this study.

The problem of numerous sheep on Mediterranean islands is documented by Fedele et al. (2022), who reported that mouflon and sheep often share the same habitat. The consequences of hybridization with the domestic form generally affect phenotypic traits (Schröder et al. 2016), tissue structure (Dumitrescu et al. 2017), and reproductive performance (Barbato et al. 2017) of mouflon. In addition to the problem of commercial sheep, feral sheep that live freely for years without human supervision present the greatest threat to the conservation, genetic integrity, resource depletion, spatial behavior, and disease transmission to the mouflon population (Iacolina et al. 2019; Brivio et al. 2022; Garel et al. 2022). The conservation impacts of feralization include intermixing with related taxons, disease vectoring, and competition (Göttert and Perry 2023). Even though our results did not show such effect taking place, the high frequency of free-ranging sheep can lead to greater introgression into mouflon, especially in the Mediterranean islands where large numbers of sheep are traditionally kept under semi-wild conditions (Brivio et al. 2022).

In summary, interaction with livestock is one of the major threats to the mouflon populations, and it may increase in many areas where two species share habitat (Satta et al. 2021; Garel et al. 2022). Finally, the Mediterranean islands are extremely rich in biodiversity and represent a genetic reservoir of species lineages, making them highly vulnerable and requiring special conservation measures to protect them (Barbato et al. 2022).

Author Contributions

Conceptualization: E.B. and N.Š.; sample providing: N.Š.; laboratory and statistical analyses E.B., T.Š.; writing—draft preparation: N.Š., E.B.; writing—review and editing: E.B., T.Š., N.Š.; funding: T.Š. and N.Š. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

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

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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