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Molecular DNA identity of the mouflon of Cyprus (Ovis orientalis ophion, Bovidae): Near Eastern origin and divergence from Western Mediterranean conspecific populations

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Research Article

Molecular DNA identity of the mouflon of Cyprus (*Ovis orientalis ophion*, Bovidae): Near Eastern origin and divergence from Western Mediterranean conspecific populations

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The mouflon population of Cyprus (*Ovis orientalis ophion*) comprises historically preserved feral descendants of sheep domesticated during the Neolithic. We determined genetic identity of this taxon in order to elucidate its systematic placement and enforce its protection. We used 12 loci of microsatellite DNA to infer genetic relationships between the Cypriot mouflon and either long-time isolated (Corsica, Sardinia) or recently introduced (central Italy) European mouflons (*O. o. musimon*). We also sequenced the mitochondrial DNA (mtDNA) Cytochrome-*b* gene to infer the origin of the Cypriot mouflon including many National Centre for Biotechnology Information (NCBI) entries of European and Near Eastern conspecifics. Microsatellites disclosed net divergence between Western Mediterranean and Cypriot mouflon. The latter was included in the highly heterogeneous Near Eastern *O. orientalis* mtDNA group, Iran representing the most credited region as the source for its ancient introduction to Cyprus. Both international and national legislation protect the mouflon of Cyprus as a wild taxon (*O. o. ophion*). However, the IUCN Red List of Threatened Species and NCBI include the Cypriot mouflon as subspecies of its respective domestic species, the sheep (*O. aries*). Unfortunately, people charged with crime against protected mouflon may benefit from such taxonomic inconsistency between legislation and databases, as the latter can frustrate molecular DNA forensic outcomes. Until a definitive light can be shed on Near Eastern *O. orientalis* systematics, we suggest that the Cypriot mouflon should be unvaryingly referred to as *O. o. ophion* in order not to impair conservation in the country where it resides.

Key words: Cyprus, domestic sheep, Mediterranean, microsatellite DNA, mitochondrial DNA, Mouflon, near eastern, *Ovis*, taxonomy, wild sheep

Introduction

The European mouflon (*Ovis musimon*, Bovidae – but see below) is thought to represent the relic of the first domesticated sheep readapted to feral life (e.g. Hiendleder, Kaupe, Wassmuth, & Janke, 2002). Historically preserved mouflon populations are presently restricted to the islands of Corsica, Sardinia and Cyprus. The conservation value of those introduced into continental Europe (in the 18th century) is varied and requires *ad hoc* investigation, as only some populations have a known history while others have multiple/mysterious origins (Andreotti et al., 2001; Boitani, Lovari, & Vigna Taglianti, 2003; Cugnasse, 1994; Piegert & Uloth, 2005; Türcke & Schmincke, 1965; Uloth, 1972).

Many revisions based on different criteria have made the systematics of the genus *Ovis* take on the appearance of a very complex puzzle (Hiendleder et al., 2002). Wilson and Reeder (2005) listed both European (*O. musimon*) and Near Eastern (*O. orientalis*) mouflon as domestic sheep (*O. aries*) subspecies (*O. a. musimon* and *O. a. orientalis*, respectively), the Cypriot mouflon being referred to as *O. a. ophion*. Other authors (e.g., Shackleton & IUCN/SSC Caprinae Specialist Group, 1997) argued

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that mouflon and domestic sheep should be considered as distinct species (*O. orientalis* and *O. aries*, respectively). Lately, DNA data suggested ranking the European mouflon as a subspecies (*O. o. musimon*) of the Near Eastern one (Rezaei et al., 2010).

Claimed for a long time as endemic to this island (Cugnasse, 1994; Hadjisterkotis, 1993, 1999; Shackleton et al., 1997), the mouflon of Cyprus (*O. orientalis ophion*) has never been included in any comparative molecular DNA study. Lawson Handley et al. (2007) investigated the genetic structure of European sheep breeds, yet only two Cypriot mouflons were genotyped at the microsatellite DNA (Short Tandem Repeats, STRs). Other authors either did not include (Rezaei et al., 2010) or used very marginally (Bruford & Townsend, 2006; Demirci et al., 2013) mitochondrial DNA (mtDNA) sequences of Cypriot mouflon. Chessa et al. (2009) investigated Eurasian sheep using endogenous retroviruses as markers and found relic genomic traits of ancestral sheep mostly in the Cypriot mouflon. While the Fertile Crescent hosted early domestication (11,000 BP), Cyprus acted as a stepping-stone since the first wave (10,500 BP) of sea-faring colonists dispersing Near Eastern livestock species westwards across the Mediterranean (Peters, von den Driesch, & Helmer, 2005; Vigne, Buitenhuis, & Davis, 1999, 2003; Zeder, 2008).

Here, in compliance with current legislation and taxonomy followed in Rezaei et al. (2010), we refer to Corsican/Sardinian and Cypriot mouflon as *O. o. musimon* and *O. o. ophion*, respectively. We attempted to determine the molecular DNA identity of the mouflon of Cyprus in order to elucidate its systematic placement and, accordingly, enforce its protection. We used a panel of STR loci to infer both genetic structure and relationships with either historically preserved (Corsica, Sardinia) or recently introduced (central Italy) populations. In addition, we employed the mtDNA to infer the origin of the Cypriot mouflon within a phylogeographic framework including many National Centre for Biotechnology Information (NCBI) entries from the Near East.

Materials and methods

The mouflon of Cyprus

In the last century, the mouflon of Cyprus faced serious challenges related to habitat loss/fragmentation, disease transmission through livestock and poaching (Ioannou et al., 2011). Population distribution range is limited to the mountainous Paphos forest (a state-owned area of about 620 km^2 managed by the Forestry Department) and adjacent forest areas in the western side of Cyprus. Census is stable (3,000 head), as recently assessed by the Game Fund Service of the Ministry of the Interior and cited in the Mouflon Management Plan (Sfouggaris, 2011). Protected by national legislation, the mouflon of Cyprus is included as *O. o. ophion* in both Annexes II/IV of 92/43 Habitats Directive and Appendix I of CITES (see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2015.1046409).

Biological sampling

The Cypriot Game Fund Service in collaboration with the Cypriot Veterinary Services collected 63 mouflon samples (dry blood spot on Whatman filter paper): 53 were from individuals captured in the Paphos forest (Fig. S1, see supplemental material online), eight from local captive animals and two of unknown origin. We also sampled 20 mouflons in Sardinia (6000 head; Apollonio, Luccarini, Giustini, Scandura, & Ghiandai, 2005) either in the wild (16: blood: Ogliastra Province) or in captivity (four: hairs: Breeding and Wildlife Recovery Centre, Bonassai, Sassari). These latter were originally from the Asinara National Park. We also collected many dry faecal samples of Corsican (1000 head, minimum; M. Garel, unpublished data) and central Italy (Tuscany) mouflons during winter (Maudet, Luikart, Dubray, Von Hardenberg, & Taberlet, 2004). Each sample was individually housed in a plastic tube, kept at 4 °C in the field and not extra dried before it was stored at −40 °C within 8h from its collection. We analysed one scat per sampling site to avoid duplicates from the same animal in both Corsican (19) and central Italy (23: Tuscan Archipelago National Park, 13; Tuscan-Emilian Apennines National Park, six; Apuan Alps Regional Park, four) populations. With the exception of faeces (no chemicals added: cf. Guerrini & Barbanera, 2009), all samples were preserved in 96% ethanol. Detailed sampling information is given in Fig. 1 and Table S1 (see supplemental material online).

DNA extraction

We extracted DNA from blood/hairs using Puregene Core Kit-A (Qiagen, Germany) and from faeces using QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. In order to minimize the risk of contamination, we thoroughly swabbed laboratory equipment with 4.2% sodium hypochlorite and autoclaved all disposables in their containers. We monitored reliability of each DNA extraction through two negative controls (no tissue added). We determined DNA concentration and purity with an Eppendorf BioPhotometer (AG Eppendorf, Germany) (faeces excluded).

Microsatellite DNA

We genotyped all Corsica, Sardinia, central Italy and Cyprus (19 + 20 + 23 + 63 = 125) samples at 12 STR

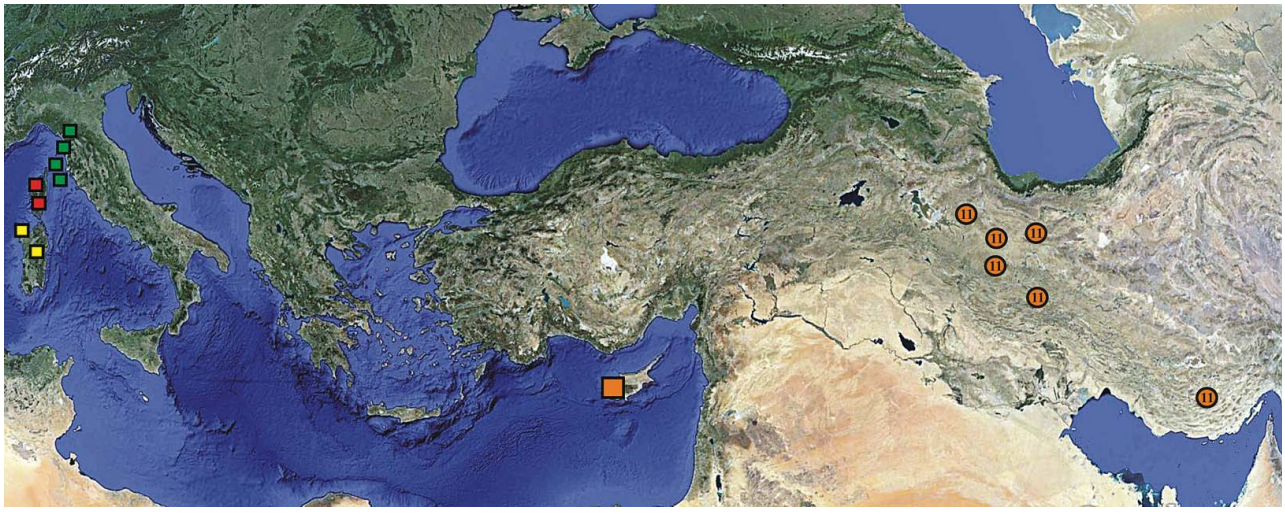

Fig. 1. Map of the study area. Red squares: Corsica (upper: Mt. Cinto population; lower: Bavella population); yellow squares: Sardinia (upper: Asinara National Park; lower: Ogliastra Province); green squares, central Italy (from the upper to the lower square: Tuscan-Emilian National Park, Apuan Alps Regional Park, Capraia Island and Elba Island); large orange square: Paphos Forest, Cyprus. Near Eastern (Iran) localities hosting H11 (the single haplotype disclosed in Cyprus, see Results) are indicated with an orange circle (cf. Fig. 4). See Table S1, online supplementary material for detailed information for each population.

loci isolated from domestic sheep (*O. aries*), goat (*Capra hircus*) and cattle (*Bos taurus*) genome (Table 1). We performed PCRs (12.5 μL) as in Barbanera et al. (2012). However, we added 0.3 μL of 1:4 diluted not-acetylated bovine serum albumin (20 mg/mL; Sigma Aldrich) to reactions including DNA from faeces/hair. We carried out gene sizing on an ABI Prism 3730 DNA automated sequencer with GENESCAN (Applied Biosystems). Only for faeces/hairs, we genotyped each locus from two to five times according to the comparative multiple-tubes approach of Frantz et al. (2003). Then, we used GIMLET (v. 1.3.3; Valière, 2002) to reconstruct consensus genotypes.

We evaluated the discriminatory power of the whole panel of loci by estimating the probability that two individuals drawn at random from the populations showed identical multilocus genotypes by chance (P_{ID} and P_{ID} sib: for the latter, we assumed sibling relationships: Paetkau et al., 1998; Waits, Luikart, & Taberlet, 2001). We used ARLEQUIN (v. 3.5.1; Excoffier & Lischer, 2010), FSTAT (v. 2.9.3; Goudet, 2001) and GENEPOP (v. 3.4; Raymond & Rousset, 1995) to (i) compute the

Table 1. Characteristics of STR loci. T_M (°C), annealing temperature; TD, touch-down PCR; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity; P_{ID}, probability that two individuals drawn at random share identical genotypes by chance; P_{ID}sib, probability of identity among siblings. STR loci are sorted according to the increasing order of their P_{ID} and P_{ID}sib single-locus values (the locus at the top is the most informative one), and a sequentially multi-loci P_{ID} (P_{ID}sib) is reported for each locus.

Locus	T_M (°C)	Size-range (bp)	Repeat motif	H_O	H_E	P_{ID}	P_{ID}sib	Literature record
OarFCB48	TD 58-55	134–168	$(GT)_{13}$	0.63	0.88	2.84×10^{-2}	3.20×10^{-1}	Buchanan et al. (1994)
ILSTS028	TD 55-50	125–175	$(AC)_{13}$	0.62	0.85	1.04×10^{-3}	1.08×10^{-1}	Kemp et al. (1995)
OarFCB304	TD 58-55	141–189	$(TC)_{7}$	0.57	0.80	6.09×10^{-5}	3.92×10^{-2}	Buchanan & Crawford (1993)
SR-CRSP8	50	211–247	$(GT)_{11}$	0.34	0.80	3.93×10^{-6}	1.44×10^{-2}	Bhebhe et al. (1994)
OarJMP58	TD 60-55	138–174	$(TG)_{18}$	0.49	0.79	2.32×10^{-7}	5.29×10^{-3}	Crawford et al. (1995)
MCM527	TD 58-55	155–179	$(GT)_{11}$	0.43	0.79	1.72×10^{-8}	1.99×10^{-3}	Hulme et al. (1994)
BM415	50	131–177	$(TG)_{13}$	0.45	0.78	1.17×10^{-9}	7.49×10^{-4}	Bishop et al. (1994)
OarAE129	TD 55-50	137–165	$(AC)_{12}$	0.30	0.76	1.08×10^{-10}	2.95×10^{-4}	Penty et al. (1993)
MAF70	TD 60-55	121–137	$(AC)_{16}$	0.33	0.69	1.31×10^{-11}	1.28×10^{-4}	Buchanan & Crawford (1992)
SR-CRSP7	50	152–192	$(GT)_n\,(AT)_n$	0.18	0.68	1.56×10^{-12}	5.64×10^{-5}	Bhebhe et al. (1994)
ILSTS011	TD 58-55	262–292	$(TC)_{9}$	0.37	0.64	2.54×10^{-13}	2.67×10^{-5}	Brezinsky et al. (1993)
SR-CRSP9	TD 58-55	99–141	$(GT)_{5}$	0.33	0.48	7.69×10^{-14}	1.57×10^{-5}	Bhebhe et al. (1994)

number of alleles per locus, the number of unique alleles and the allelic richness; (ii) calculate expected (H_E) and observed (H_O) heterozygosity; (iii) infer deviations from both Hardy−Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LE) (10,000 dememorization, 100 batches, 5,000 iterations per batch); (iv) investigate the partition of the STR diversity within and among populations by AMOVA; (v) infer the degree of genetic differentiation among populations by estimating average pairwise F_{ST} distance values. These latter were also plotted on the first two axes of a Principal Components Analysis (PCA) using STATISTICA 5.0/W (Statsoft Inc., USA). We adopted Bonferroni correction (Hochberg, 1988) to adjust the significance level of each statistical test.

Bayesian clustering analysis was performed with STRUCTURE (v. 2.3.4; Pritchard, Stephens, & Donnelly, 2000) to investigate the spatial structure of the genetic diversity. We attempted to determine the K (unknown) clusters of origin of the sampled individuals and to assign them to each cluster. Simulations were performed with 10^6 Markov Chain of Monte-Carlo iterations (burn-in: 10^5 iterations) and replicated five times per each K-value (1 to 12). We choose the correct K-value using the maximum of the function $\Delta K = m(|L(K+1) - 2\,L(K) + L(K-1)|)/s[L(K)]$, where $L(K)$ stands for 'log estimated likelihood' calculated for each K value, m for "mean" and s for "standard deviation" (Evanno, Regnaut, & Goudet, 2005). An identification threshold (Q_i) to each cluster was set to 0.90 (Väha & Primmer, 2006).

Only for the Cypriot mouflon population, we computed maximum likelihood estimates of relatedness (i.e., the likelihood that a pair of individuals would be classified as either full-siblings, half-siblings or unrelated) with ML-RELATE (Kalinowski, Wagner, & Taper, 2006), and we calculated the inbreeding coefficient (f; Weir & Cockerham, 1984) using GENETIC DATA ANALYSIS (v. 1.1) (1,000 bootstrapping replicates across loci). We used BOTTLENECK (v. 1.2.02: Piry, Luikart, & Cornuet, 1999) with a Two Phase Mutation (TPM) model (1,000 replicates; Di Rienzo et al., 1994) to find evidence of genetic bottlenecks, and carried out a qualitative mode signed-rank test.

Mitochondrial DNA

We amplified the entire mtDNA Cytochrome-*b* gene (Cyt-*b*, 1140 bp) using primers Cytb_F and Cytb_R of Pedrosa et al. (2005). PCR (50 μL) reactions contained 1 μL of Ampli*Taq* Gold DNA Polymerase (1 U/μL), 4 μL of 25 mM $MgCl_2$, 5 μL of 10 × PCR Gold buffer (Applied Biosystems, USA), 5 μL of 2.5 mM dNTP (Sigma Aldrich, Italy), 3 μL of each primer (1 μM) and *c*. 20 ng of DNA template (for faeces: 3 μL, final elution). We performed PCRs in a MyCycler thermal cycler (v. 1.065, Biorad) with the following profile: 3 min 94 °C, 35 cycles of 1 min 94 °C, 2 min at 55 °C and 1 min 72 °C, followed by 7 min 72 °C. For faecal samples only, however, when we could not visualize any PCR product after the gel electrophoresis, we re-amplified first amplicon in a semi-nested PCR as described by Guerrini and Barbanera (2009). PCR products were purified (Genelute PCR Clean-up Kit, Sigma Aldrich) and directly sequenced on both DNA strands using the BigDye Terminator v. 3.1 Cycle Sequencing Kit on an ABI 3730 DNA automated sequencer (Applied Biosystems).

We sequenced the Cyt-*b* gene for 41 Cypriot and all remaining (Corsica + Sardinia + central Italy: 62) samples (41 + 62 = 103). In order to include in the alignment 57 GenBank entries (Corsica, two; Turkey, nine; Armenia, one; Iran, 45: Table S1, see supplemental material online) we cut our sequences at both 5'- (positions: 1−21) and 3'- (positions: 1064−1140) ends. Hence, we aligned 160 sequences (final length: 1042 bp) with CLUSTALX (v. 1.81: Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and inferred haplotype composition with DNASP (v. 5.00: Librado & Rozas, 2009).

We selected the HKY85 (Hasegawa, Kishino, & Yano, 1985) + I + G substitution model using MODELTEST (v. 3.06: Posada & Crandall, 1998) and the Akaike Information Criterion (AIC = 3945.1; Akaike, 1974). Then, we performed a Maximum Likelihood (ML) tree reconstruction using PHYML (v. 3.0: Guindon et al., 2010) platform (www.atgc-montpellier.fr) and setting main parameters as follows: I = 0.77, α = 0.017 and T_i/T_v = 7.50. We employed *O. ammon argali* (Argali or mountain sheep) AJ867266 sequence of Bunch, Wu, and Zhang (2006) as outgroup, and evaluated the statistical support for each node by bootstrapping (BP, with 10,000 replicates: Felsenstein, 1985). We also constructed a haplotype network with DNA ALIGNMENT (v. 1.3.3.2; 2003−2013 Fluxus Technology) and the Median Joining method (Bandelt, Forster, & Röhl, 1999) as in NETWORK (v. 4.6.1.2; 2004−2014 Fluxus Technology). We excluded Armenia from our dataset (one sequence: Table S1; see supplemental material online) before using ARLEQUIN to calculate haplotype diversity (h), mean number of pairwise differences (k), and nucleotide diversity (π) for each population. The AMOVA was performed among and within the populations using the ϕ_{ST} analogous to Wright's (1965) F-statistics (1,000 permutations).

Results

Microsatellite DNA

The STR panel was powerful in discriminating individuals (n = 125: $P_{ID} = 7.69 \times 10^{-14}$ and $P_{ID}sib = 1.57 \times 10^{-5}$; Table 1), as values lower than 0.001 can be considered satisfactory (Waits et al., 2001). All loci were highly polymorphic with the exception of SR-CRSP7 and SR-CRSP9

Table 2. STR genetic variability for each Mediterranean population: n, sample size; n_a, average number of alleles/locus; A_r, allelic richness; A_u, number of unique alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; p_{HWE}, probability value for the Hardy–Weinberg Equilibrium test; χ^2 test with relative degrees of freedom (*df*) (Fisher exact test, all loci). Departure from HWE was significant in all populations after Bonferroni correction ($\alpha = 0.05$, $\alpha' = 0.05/48 = 0.001$).

Population	n	n_a	A_r	A_u	H_O	H_E	p_{HWE}	χ^2 (*df*)	Average gene diversity
Corsica	19	7.9	7.7	21	0.54	0.77	< 0.001	∞ (24)	0.69
Sardinia	20	5.4	5.2	8	0.55	0.66	< 0.001	81.8 (24)	0.61
Central Italy	23	7.2	6.8	14	0.48	0.74	< 0.001	∞ (24)	0.64
Cyprus	63	5.5	3.6	30	0.39	0.49	< 0.001	∞ (20)	0.39

(monomorphic within the Cypriot population). The total number of alleles at each locus ranged between nine and 15 (13.3, on average): the mouflon of Cyprus hold either the lowest average number of alleles per locus (5.5) or the highest total number of private alleles (30) (Table 2).

All populations showed significant departure from HWE due to heterozygote deficiency after Bonferroni correction (Fisher test: $P < 0.001$: Table 2). Such deviation was highly significant at four, three and five loci in Cyprus, Corsica and central Italy, respectively ($p < 0.001$, Table S4, see supplemental material online). Average level of both H_O and H_E (0.39 and 0.49, respectively: Table 2) was lower in the Cypriot mouflon populations than in all of the other ones. LE test carried out for all pairs of loci across all populations was significant only for one (MAF70 versus OarJMP58) in 45 comparisons, only in the population from Corsica ($P < 0.001$, $P < \alpha' = \alpha/180 = 0.05/180 = 0.0003$, after Bonferroni correction) (data not shown).

We found that 66.2% of the STR variability was partitioned within populations and 33.8% among them ($F_{st} = 0.34$, $p < 0.001$). In the PCA plot (Fig. 2, upper part), the first two components explained the 98.2% of the total variability. The Cypriot population diverged from all the western Mediterranean ones ($0.38 < F_{st} < 0.47$, $p < 0.001$: Table 3), while mouflons of Corsica and central Italy ($P = 0.11$: Table 3) were closer to each other than to Sardinia (Fig. 2 and Table 3).

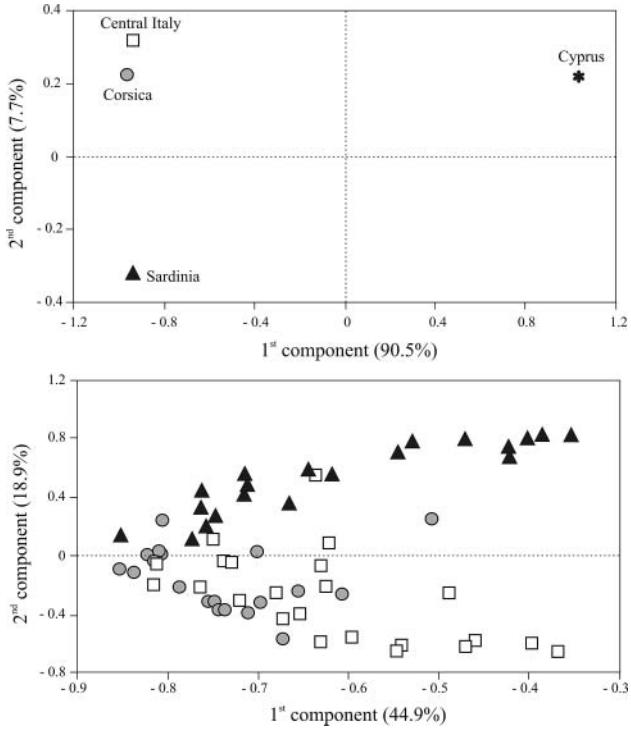

Fig. 2. The Principal Component Analysis performed using average pairwise F_{ST} distances among STR genotyped populations (upper part) and single mouflons (lower part: Cyprus excluded). The percentage of total variance explained by each of the first two components is given. Symbols are the same in both parts.

Table 3. Above diagonal: average pairwise distance values (F_{ST}) computed for the STR genotyped populations. Below diagonal: average pairwise distance values (ϕ_{ST}) computed among mtDNA genotyped populations (Armenia was excluded as it includes only one GenBank entry). All *p* values were highly significant ($p < 0.001$) except for Iran versus Turkey ($P = 0.01$) and Corsica versus Central Italy ($P = 0.11$) comparisons.

	Corsica	Sardinia	Central Italy	Cyprus	Turkey	Iran
Corsica	–	0.127	0.064	0.392	–	–
Sardinia	0.329	–	0.147	0.465	–	–
Central Italy	0.044	0.331	–	0.384	–	–
Cyprus	0.956	0.973	0.947	–	–	–
Turkey	0.612	0.629	0.607	0.810	–	–
Iran	0.555	0.520	0.559	0.387	0.112	–

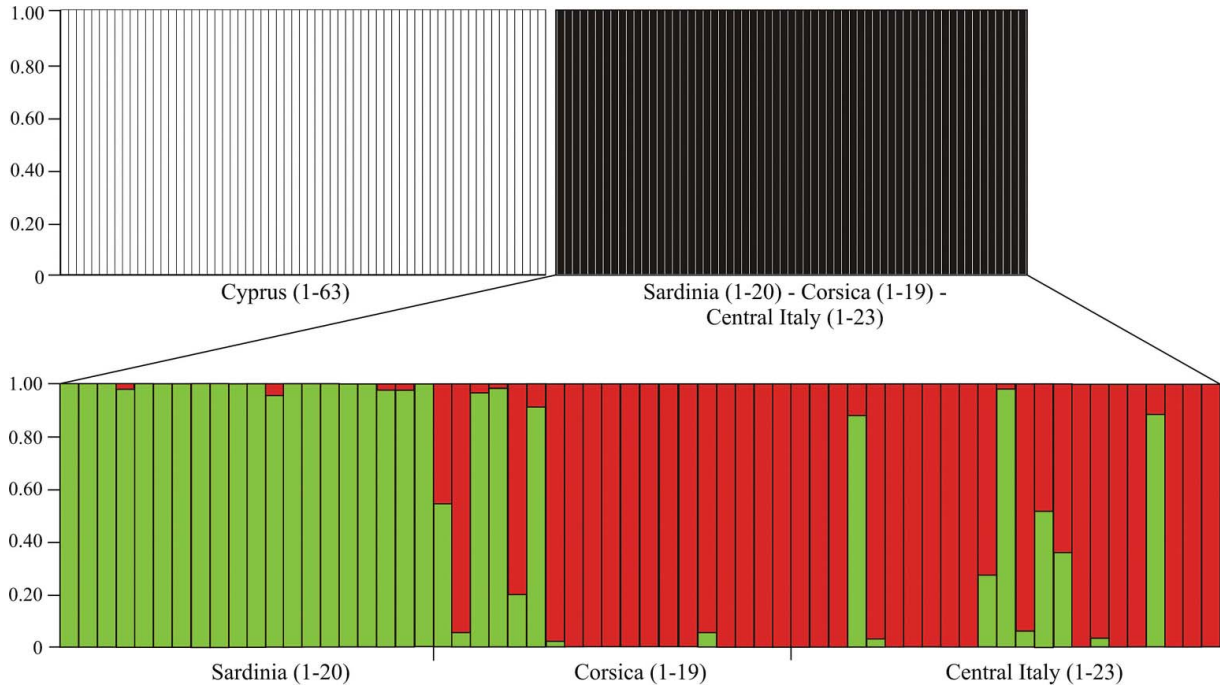

Fig. 3. Bayesian admixture analysis of STR genotypes computed by STRUCTURE with $K = 2$. Upper part: all populations. Lower part: only Sardinia, Corsica and central Italy. Each individual is represented as a vertical bar partitioned in K segments, whose length is proportional to the estimated membership in the K clusters.

Some deviation from HWE notwithstanding, we felt confident in using the entire STR panel for all individuals in the Bayesian clustering analysis, as it has been proved this may have a negligible effect on simulated assignment tests (Cornuet, Piry, Luikart, Estoup, & Solignac, 1999). In the STRUCTURE analysis mouflons were partitioned into two groups: the first included all individuals from Cyprus, while the second all those from Corsica, Sardinia and central Italy (Fig. 3, upper part: $Q_I = 1.00$, all populations). We repeated the analysis excluding the Cypriot population. We found that all mouflons from Sardinia were assigned to the cluster I ($Q_I = 0.99$: Fig. 3, lower part). Corsica and central Italy hold low assignment value to cluster II ($Q_{II} = 0.80$ and 0.82, respectively: data not shown), as their individuals clustered into group II (Corsica: 14; central Italy: 17), I (Corsica: three; central Italy: one) or were admixed (Corsica: two; central Italy: five).

As far as the population of the island of Cyprus is concerned, the PCA carried out using STR data from each sampling locality marked out a slight longitudinal gradient of genetic differentiation across the Paphos forest (Fig. S1, see supplementary material online). Nonetheless, the Bayesian clustering analysis did not confirm this result (see below). Coming to the single Cypriot mouflons, the average pairwise relatedness ranged from zero (1014 pairs) to one (one pair). We found that 78.5% of individuals were unrelated (1891 comparisons), 11.7% half siblings, 5.2% parent/offspring and 4.6% full siblings; the value of the coefficient of inbreeding (f) was 0.190. The frequency distribution of the STR alleles (Fig. S2, see supplementary material online) as well as all tests that were performed (Table S2, see supplementary material online) did not point to the occurrence of genetic bottlenecks.

Mitochondrial DNA

We found 36 haplotypes (H1-H36; accession codes: LN651259- LN651268, Table S1, see supplementary material online). The Iranian population showed the highest value for all diversity indexes, whereas one haplotype (H11) only was disclosed in Cyprus (Fig. 4, Table S3, see supplementary material online). The 66.4% of the variability was partitioned among populations while the 33.6% within them ($\phi_{ST} = 0.66$, $p < 0.001$).

ML tree and network concordantly disclosed two main groups of haplotypes. In the phylogenetic reconstruction (Fig. 4), first clade (BP = 80) included western European mouflons from Corsica, Sardinia and central Italy, one *O. orientalis gmelini* from Iran (H7) being the only exception. However, several Turkish and Iranian individuals shared haplotype H1 (see also Table S1, see supplementary material online), which was sister lineage to the previously mentioned group. Second clade (BP = 85) included most of Near Eastern *Ovis orientalis* ssp. In particular, the single Cypriot mouflon haplotype (H11) fell into a sub-clade (BP = 77) including mostly Iranian

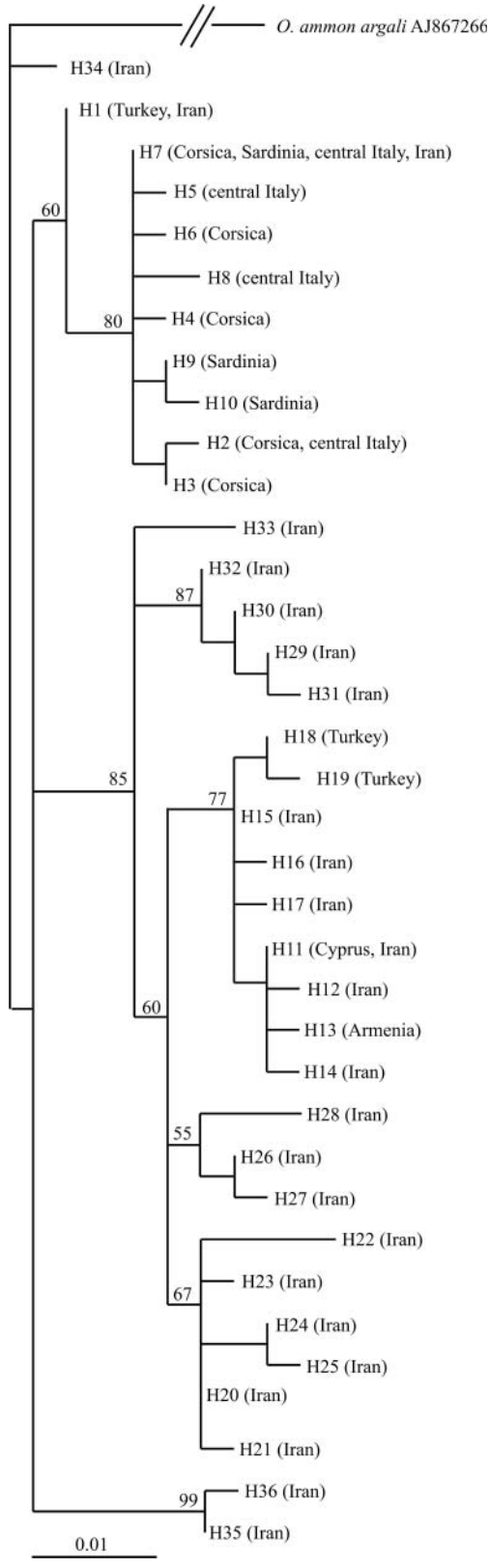

Fig. 4. ML tree computed by PHYML for the aligned haplotypes (H) and using *O. ammon argali* as outgroup. Statistical support (bootstrapping percentage) was reported above each node. Scale bar is proportional to the number of substitutions per site.

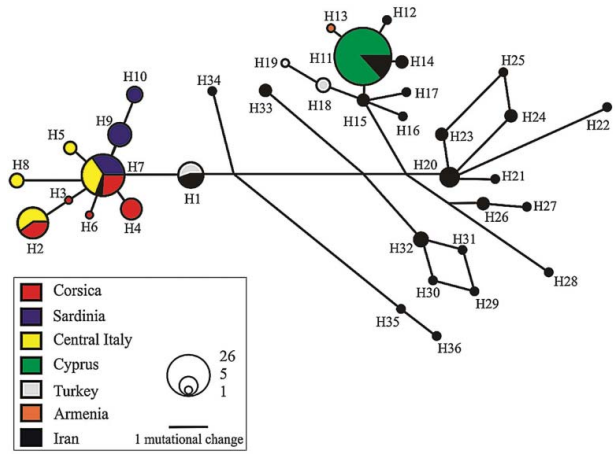

Fig. 5. Haplotype network. A scale to infer the number of haplotypes for each pie is provided together with a length bar to compute the number of mutational changes.

mouflons (with some Turkish/Armenian individuals). In the network (Fig. 5), first cluster included haplotypes (H11−H36) held by mouflons from Cyprus, Turkey, Armenia and Iran. Six Iranian individuals (*O. o. gmelini* and *O. o. laristanica* from North West and South Iran, respectively: Fig. 1 and Table S1, see supplementary material online) shared single Cypriot haplotype (H11), while others from Iran and Armenia (private H13) were only one mutational step away from the latter. In the second cluster (H2−H10), which included all *O. o. musimon* individuals, haplotype H7 was shared by all West Mediterranean populations and one Iranian mouflon (Table S1, see supplementary material online). Haplotype H1 lay between the two clusters and included both Turkish and Iranian mouflons with various taxonomic assignations (Demirci et al., 2013; Rezaei et al., 2010; Table S1, see supplementary material online). We reported in Table 3 the ϕ_{ST} distance values obtained from all population pair comparisons.

Discussion

With the exception of a preliminary investigation carried out in Corsica (Maudet & Dubray, 2002), this study represents the first survey on Mediterranean mouflon populations relying on a panel of microsatellite DNA loci. Principal Component Analysis of STR variability, Bayesian clustering of individual multilocus genotypes, and average F_{ST} pairwise distance values computed among all population pairs concordantly disclosed net genetic separation between the mouflon of Cyprus and those from the western Mediterranean (Figs 2, 3 and Table 3). Among these, Corsican and central Italy populations were much more closely related to each other than to Sardinian ones,

which diverged from both of them. Although confirmed by mtDNA ϕ_{ST} distance values computed among all population pairs (Table 3), such a result was unexpected. The very large majority of mouflons introduced into the Italian Peninsula after the 1970s (e.g., all sampled populations of this study: Table S1, see supplementary material online) were originally from the Wildlife-Hunting Company of Miemo (Tuscany) (Masseti, 2003). Here, a balanced stock of Sardinian and Corsican mouflons was kept in captivity since the 1960s. While the export of mouflons from central Italy to Corsica can be excluded, it sounds possible that present-study small sample sizes available for each area in central Italy (Tuscan-Emilian Apennines, Apuan Alps, Elba Island, and Capraia Island: Table S1, see supplementary material online) have probably allowed for a non-random sorting of Corsican versus Sardinian genotypes. Nevertheless, in the mountain habitat where all these sampled populations were introduced about 40 years ago, selection might have also differently shaped genetic diversity of descendants of Corsican/Sardinian source stocks. Kaeueffer, Coltman, Chapuis, Pontier, and Denis Réale (2007), for instance, attributed to selection an unexpectedly high level of heterozygosis found in a sub-Antarctic island mouflon population established in 1957 by a single pair of captive French individuals.

There is a huge body of evidence that diversity can be rapidly lost in small populations because of genetic drift and related inbreeding (e.g., Reed & Frankham, 2003). In the Mediterranean mouflons, geographic partition of mtDNA diversity was much larger than that disclosed at microsatellite DNA loci. The ratio of mtDNA ϕ_{ST} to microsatellite F_{ST} was, indeed, $0.66/0.34 = 1.95$. Contrasting results between the two genetic systems can be attributed to the fact that the effective population size of mtDNA genome is 1/4 of that of the nuclear DNA (Birky, Fuerst, & Maruyama, 1989). Decline in mtDNA diversity can be much faster in fragmented populations or, similarly, in those derived from a few founders. Hence, comparatively low nuclear and null mitochondrial DNA diversity of the Cypriot mouflon did not come as a surprise (Table 2 and Table S3, respectively, see supplementary material online). This population has been isolated for thousands of years, as there is no evidence for further introductions since the Neolithic. However, neither average pairwise relatedness nor inbreeding coefficient values disclosed in this study arouse concern over the long-term survival of such population. On the contrary, although detected only by some molecular tools (cf. Fig. 3 versus Fig. S1, see supplementary material online), evidence of population genetic structure was found across the Paphos forest. Furthermore, it is known that in the 1930s hunting pressure had reduced the mouflon population of Cyprus to only about 20 individuals (<1% of the present-day population; Forestry Department, 2012; Maisels, 1988). It is worth recalling here that only severe reduction in the population size (at least by 100-fold) can be detected by software such as BOTTLENECK using a number of STR loci comprised between 10 and 20 (Cristescu, Sherwin, Handasyde, Cahill, & Cooper, 2010). In the present study, however, three tests for mutation drift equilibrium relying on 12 STR loci did not disclose any sign of a genetic bottleneck in the Cypriot mouflon (Table S2, see supplementary material online). We also analysed the frequency distribution of STR alleles, which allows detection of a bottleneck efficiently when it has occurred 2-4 $\times$ N_e generations ago (N_e, effective size, i.e., the number of reproductive mouflons). Setting $N_e = 10$ for the mouflon population in the 1930s, time elapsed would have definitely allowed for disclosure of genetic bottlenecks. Once more, high frequency of rarest STR alleles strongly pointed against such an occurrence (Fig. S2, see supplementary material online).

About 11,000 years BP, at the onset of the very first wave of human-mediated dispersal of livestock across the Mediterranean Basin, the island of Cyprus acted as a staging ground for introductions towards western regions, which indeed were reached by such expansion only a few thousands of years later (Guilaine, 2003; Masseti, 1997; Zeder, 2008). According to this, Chessa et al. (2009) found relic genomic traits of ancestral sheep mostly in the Cypriot mouflon. We realize that present-day genetic structure of Mediterranean mouflon populations represents the outcome of many historical events. Nonetheless, we found the lowest value of both nuclear (Table 2) and mitochondrial (Table S3, see supplementary material online) DNA diversity in the mouflon of Cyprus, while the highest ones were disclosed in the populations of Corsica and Sardinia. Such a pattern closely resembles that discovered by Pereira, van Asch, Bradley, and Amorim (2005, 2006) in both Mediterranean sheep and goats. These authors found unexpected high genetic diversity at the westernmost periphery of the Mediterranean Basin, in Portugal, and attributed the latter to multiple introductions of caprinae into the Iberian Peninsula (Zeder, 2008). Furthermore, our mitochondrial DNA results (Figs 4, 5) pointed to the subdivision of *Ovis orientalis* into two groups (cf. Valdez, 1982), the first including *O. o. musimon* from Corsica, Sardinia and central Italy, and the second a few *O. orientalis* morphological subspecies (also *O. o. ophion* from Cyprus, see below) from the Near East. More importantly, both mtDNA tree and network acknowledged the ancestral position of H1 (Turkey, Iran) and H7 (Iran) haplotypes with respect to the western *O. orientalis* group, as well as H34−H36 (Iran) intermediate placement between Near Eastern and western *O. orientalis* group. As to the latter, genetic drift (e.g., see long branches for H35 and H36: Figs 4, 5) likely affecting small populations could also be assumed. Overall, mtDNA suggests that *O. o. musimon* derived from the Near Eastern *O. orientalis* group, present-day mouflons

still retaining a few oriental haplotypes along a westwards decreasing gradient across the Mediterranean. MtDNA also pointed to North West Iran as the most credited geographic region as the source for its ancient introduction to Cyprus. This result was in agreement with genetic data of Bruford and Townsend (2006) and the known archaeozoological pattern for livestock domestication and diffusion across the Mediterranean (Zeder, 2008). In spite of the geographic range reported for *O. gmelinii* (= *O. o. anatolica*, *O. o. gmelini*, *O. o. isphahanica* and *O. o. laristanica*) by Demirci et al. (2013), however, we could not untangle Cypriot mouflon (*O. o. ophion*) identity as well as any of the members of its group (Figs 4, 5).

Cypriot mouflon conservation: systematics, legislation and DNA database

Systematics of the genus *Ovis* is a very complex matter. Wild sheep found on Mediterranean islands are recognized as introduced by humans. Some authors (e.g., Gentry, Clutton-Brock, & Groves, 2004; Gippoliti & Amori, 2004; Wilson & Reeder, 2005) proposed to include them in the domestic species (*O. aries*) and not as subspecies in wild taxa. However, Rezaei et al. (2010) drawn a mtDNA phylogenetic picture where the majority of the morphological species of Nadler et al. (1973) were confirmed and Mediterranean mouflons figured as *O. orientalis* (cf. Shackleton et al., 1997). On the other hand, taxonomic information not merely pertains to systematics and evolution as such but also to conservation management. If, on the one hand, DNA-based studies lead sometimes to taxonomic over-splitting and species inflation (e.g., Groves & Grubb, 2011), on the other hand they have warranted long-awaited breakthroughs in the knowledge and protection of biodiversity (e.g., Zachos et al., 2013; Zachos, Mattioli, Ferretti, & Lorenzini, 2014). In addition, rapid growth of forensic DNA analysis in crimes against protected wildlife made uniform recording of taxonomic information in legislation and DNA databases inevitable (Alacs, Georges, FitzSimmons, & Robertson, 2010; Iyengar, 2014). DNA sequences, indeed can be of high relevance in court cases and the genus *Ovis* is not an exception in this regard (Barbanera et al., 2012; Lorenzini, Cabras, Fanelli, & Carboni, 2011).

The mouflon of Cyprus is included as subspecies of either wild (*O. o. ophion*) or domestic (*O. aries*) within international (Habitats Directive, CITES) as well as national legislation and the IUCN Red List of Threatened Species plus NCBI database (based on Wilson & Reeder, 2005), respectively. Regardless of the IUCN use of 'Cyprus mouflon' as the common name for vulnerable *O. orientalis*, Cyprus is not included in the geographic range of the species nor is *O. o. ophion* reported in Valdez (2008). Unfortunately, quotation of Cypriot mouflon DNA entries under *O. aries* represents an Achilles heel in court cases, as taxonomic inconsistency between NCBI and national legislation may favour people charged with crime against protected wildlife by frustrating molecular DNA forensic outcomes (Barbanera et al., 2012) and undermining conservation efforts to protect the species.

In this study, microsatellite DNA disclosed significant divergence between West Mediterranean *O. o. musimon* and the Cypriot mouflon. The latter was included in a mtDNA group with *O. o. anatolica*, *O. o. gmelini*, *O. o. isphahanica* and *O. o. laristanica* individuals. MtDNA also pointed to the introduction of the mouflon from Iran to Cyprus. However, lack of Iranian samples prevented us from testing at the microsatellite DNA level whether long-time isolation eventually allowed the Cypriot mouflon to diverge from its source population. On one hand, we recommend this investigation be highly prioritized as it can certainly convey further conservation value to the mouflon of Cyprus. On the other hand, until definitive light can be shed on taxonomically heterogeneous Near Eastern *O. orientalis* group, we suggest that the mouflon of Cyprus should be unvaryingly acknowledged as *O. orientalis ophion* not to impair conservation in the country where it resides. In the light of the genetic divergence disclosed between Cypriot and European mouflon, we also recommend to ban importation of any mouflon into Cyprus to preserve the integrity of the island population.

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Supplemental data

Supplemental data for this article can be accessed here.

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