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## Phylogeographic and population genetic structure of hound-like native dogs of the Mediterranean Basin

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## ABSTRACT

The dog was probably the first domesticated animal. Despite extensive archaeological and genetic investigations, the origin and the evolution of the extant dogs are still being debated. Dog breeds that have over time been selected for hunting share common ancestral traits. This study represents the first comprehensive attempt to survey at the genomic and mitochondrial level eight hound-like dogs breeds indigenous to the Mediterranean Basin to determine if they share common ancient origins.

Results from the microsatellite analysis indicate that all the dog populations have a low inbreeding value. The Kelb tal-Fenek has a high divergence from the current Egyptian street population, however there is not enough evidence from this study to exclude completely the potential of an ancient common relationship. Overall, the mitochondrial results indicate high frequencies of haplogroups A and B and a low representation of haplogroup C, while only one Egyptian dog could be assigned to haplogroup D. Results reveal identities and shared clades, suggesting the conservation of ancient European mitotypes in the Mediterranean hound-like breeds, especially in the Egyptian population.

Although none of the dog populations/breeds participating in this study indicate to be direct descendants of the Egyptian dogs, they still have a very close morphologically resemblance to those iconic Egyptian dogs often depicted in ancient art forms and share some genetic links with the current Egyptian population. Further research is required with other markers such as complete mitogenomes and SNP panels to confirm the complex history of the Mediterranean dogs involved in this study.

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## 1. Introduction

Apart from being the first species to be domesticated, the dog (*Canis lupus familiaris*) was also trained by humans to perform specific complementary roles such as a guard, a hunting aid, and also as a companion. Despite numerous efforts to study the phylogeny of the domestic dog, the origins and evolution of extant breeds are still a controversial issue (Wang et al., 2016; Freedman and Wayne, 2017; Bergström et al., 2022). One of the proposed theories on the geographical origin of the domestic dog is that they originated in Southwest Asia, presumably in the Fertile Crescent some 15,000 years ago (Clutton-Brock, 1981). However, Brassard et al. (2022) reports that the majority of extant dog breeds were developed during the last 200 years (defined as ‘modern’ times). The most investigated breeds with regards to ancient dog mitochondrial DNA (mtDNA) are the European dog populations (Frantz et al., 2016; Botigué et al., 2017; Yankova et al., 2019). The initial investigations of modern dogs through maternally transmitted mtDNA indicated the place of origin as being the southern part of East Asia (Pang et al., 2009; Savolainen et al., 2002). Further analyses of archaeological and genetic records focused on the possibility of Europe being at least a secondary domestication centre (Thalmann et al., 2013). Recent scientific findings have unveiled that the oldest evidence of dogs in the Mediterranean to be reconducted to Late Pleistocene in Italy (Boschin et al., 2020; Koupadi et al., 2020), hence proposing that the southern part of Europe as an important ancient source of domestication dynamics. Nevertheless, scholars are still debating on where dog domestication occurred, and whether it happened at multiple occasions or independently in various locations (Shannon et al., 2015; Frantz et al., 2016; Wang et al., 2016; Ollivier et al., 2018; Bergström et al., 2022). Once dogs were domesticated, human-mediated selection for specific traits that best fit the different requirements led to the gradual shaping and moulding of the extant breeds. Undoubtedly, the Mediterranean region, and the Near East have played a key role in replacing the original indigenous dog types with improved breeds (Pilot et al., 2015). This practice can be traced back to the Late Antiquity and the Medieval period (Marinov et al., 2018; Yankova et al., 2019). The emerging picture shows a very complex post-domestication history of the dog, which was just as eventful as the history of humans.

Nowadays, the ‘Fédération Cynologique Internationale’ (FCI) identifies and recognises 360 different canine breeds, which vary considerably in morphology, physiology, and behaviour, and are classified in ten ethnological groups ([www.fci.be](http://www.fci.be)). Group five categorises ‘Spitz and primitive type’ and is further subdivided into seven sections which includes several Mediterranean breeds. Section 6 (‘Primitive type’) includes the Kelb tal-Fenek, while section 7 (‘Primitive type-Hunting Dogs’) includes two Spanish breeds (Podenco Canario and Podenco Ibicenco), one Portuguese breed (Podengo Português, also known as Portuguese Warren Hound), and one Italian breed (Cirneco dell’Etna). The Royal Spanish Canine Society ([www.rsce.es](http://www.rsce.es)) also recognises and includes the Podenco Andaluz and the Podenco Valenciano (which are not recognised by the FCI) in section 5 ‘Hunting Dogs’ of the National Origins Book. All these breeds are morphologically quite similar, probably due to their being subjected to human mediated selection to enhance similar hunting traits, the common geographical area in which they live (Mediterranean Basin) and their proximate genetic kinship. The Kelb tal-Fenek breed, is also known as Pharaoh Hound due to its sighthound-like appearance, the elegant and elongated head and their prick and alert ears all similar to the depictions of ‘Anubis’ (ancient Egyptian God of the Death) and of the ‘Tesem’ (sighthound-like type of dogs from ancient Egypt). Based on the hypothesis that there is a connection between the ‘Tesem’ hound of ancient Egypt and today’s Kelb tal-Fenek, the modern Egyptian Baladi street dog, that descend from a mixture of ancient Egyptian dogs such as Pharaoh Hounds, Salukis, and Canaan dogs (Elmadawy et al., 2019; El Shazly, 2019), was included in this study. Indeed, the Cirneco dell’Etna has already been investigated together with the Kelb tal-Fenek, using SNP analysis

(Cortellari et al., 2021), and results indicate that the two breeds are genetically close. Moreover, all the aforementioned selected populations/breeds included in this study show a close morphological association with dogs depicted in Ancient Egypt painting (Supplementary Table S1).

In order to assess the current genetic relationships and the phylogenetic history of the selected breeds, two genetic markers, microsatellites and mitochondrial DNA (mtDNA) were studied. Canine microsatellites have already been successfully utilised to investigate the genetic structure and diversity of dog breeds by many authors (Leroy et al., 2009; Parker, 2012; Bigi et al., 2015; Sechi et al., 2016). However, modern technologies such as SNP and whole genome data have demonstrated more accurate estimates of population-level diversity, greater power to identify groups in clustering methods, and the ability to consider local adaptation (Soh et al., 2021; Yu et al., 2022). Similarly, mtDNA has been widely employed to estimate genetic diversity, phylogenetic relationships, and maternal origins among closely related modern populations. For such purposes, the D-loop hypervariable region 1 (HV1), which is the most variable part of dog mtDNA, has been widely exploited to establish the relationship among different dog breeds, and consequently, expose their origin (van Asch et al., 2013). Six main mitochondrial groups labelled from A to F characterize the dog phylogeny across the globe: A, B, and C are the most widely distributed haplogroups (Hg) among recent dog populations, while D, E, and F show a restricted geographic distribution (Duleba et al., 2015).

The aim of this study is to bring to light the genetic relationships that may exist between the eight selected hound-like dog populations/breeds at the genomic and mitochondrial level, and to determine if they share a common ancient ancestral origin.

## 2. Material and methods

### 2.1. Sample collection

Samples (blood or hair) were randomly collected from 272 genetically unrelated dogs from eight Mediterranean indigenous dog populations/breeds: four Spanish native breeds (Podenco Valenciano,  $N = 61$ ; Podenco Ibicenco,  $N = 38$ ; Podenco Canario,  $N = 30$ ; Podenco Andaluz,  $N = 31$ ), one Portuguese breed (Podengo Português,  $N = 17$ ), one Maltese breed (Kelb tal-Fenek,  $N = 32$ ), one Egyptian population (Egyptian Baladi street dog,  $N = 30$ ), and one Italian breed (Cirneco dell’Etna,  $N = 33$ ). Five of the selected breeds (Podenco Ibicenco, Podenco Canario, Podengo Português, Kelb tal-Fenek, and Cirneco dell’Etna) are internationally recognised by the FCI, and classified in group 5, section 6 (Primitive type) and in section 7 (Primitive type – Hunting Dogs). The details of the studied dog populations are reported in Supplementary Table S1. Twenty-nine German Shepherd individuals were also included as outgroup for the microsatellite investigation.

Blood samples were collected in tubes contain EDTA as an anticoagulant and stored at  $-20\text{ }^{\circ}\text{C}$  until analyses, whereas hair samples were picked from the dorsal area, kept in sealed plastic bags, and maintained at  $4\text{ }^{\circ}\text{C}$  until processing. The genomic DNA was extracted from blood samples using the GenElute™ Mammalian Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA), while the Chelex 100-based method (Walsh et al., 1991) was used for the hair samples (ten selected hair roots for each animal). Afterward, quantification and qualification of DNA was checked using NanoDrop 2000 (Thermo Scientific, Waltham, MA). Genotyping was performed by two laboratories specialising in parentage testing, ABC S.L. (Animal Breeding Consulting S.L., Cordoba, Spain) and BioGene (Catania, Italy). The two laboratories have participated in the comparison tests of the International Society of Animal Genetics - ISAG (<https://www.isag.us/comptest.asp>) and used appropriate internal standards that make their results comparable.

The experimental protocol used in this study was approved by the Ethical Committee of the University of Messina, Italy (No. 040/2020). The international rules and guidelines for animal experiments were

rigorously adopted and adhered to.

## 2.2. Microsatellite analysis

A panel of sixteen microsatellite markers, as recommended by ISAG and routinely used for canine parentage testing, were selected for this study: AHTk211, REN162C04, INU055, AHT137, FH2054, INU030, AHT121, REN169D01, REN247M23, AHTh260, REN54P11, INRA21, CXX279, AHTk211, REN169O18, and INU005 (Supplementary Table S2).

The total number of alleles per locus ( $N_a$ ), observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity, polymorphic information content (PIC) per locus and mean number of alleles (MNA) per breed were assessed using MICROSATELLITES TOOLKIT software (Park, 2001).

The rarefaction method implemented in HP-RARE version 1.0 software (Kalinowski, 2005) was used to calculate the average allelic richness (Rt) for each population/breed, using a sample of five individuals. Fisher's exact test for establishing Hardy-Weinberg equilibrium (HWE) across loci and breeds was estimated with GENEPOP package version 4.0.10 (Rousset, 2008). The levels of significance were adjusted for multiple pairwise comparisons by using the false discovery rate correction (Benjamini and Hochberg, 2000). The distribution of gene variation within and between populations was estimated, according to the Wright  $F$ -statistics using Weir and Cockerham's method (Reynolds et al., 1983).  $F_{IS}$  for each population was calculated via bootstrap using 1000 replicates with GENETIX software version 4.05 (Belkhir, 2004). The pairwise  $F_{ST}$  values were estimated according to Weir and Cockerham (1984), and as implemented in Arlequin 3.5.2 (Excoffier and Lischer, 2010). Ten thousand random permutations were used to test significance, and  $\alpha$  for each test was adjusted for multiple comparisons using the modified false discovery rate (FDR) method (Benjamini and Hochberg, 2000). Reynolds' genetic distances (Reynolds et al., 1983) between all pairs of dog populations were calculated with Arlequin 3.5.2 (Excoffier and Lischer, 2010) and used to construct a network of breed relationships following the Neighbor-Net method in SplitsTree4 4.13 (Huson and Bryant, 2006).

Hierarchical analysis of molecular variance (AMOVA) with 10,000 permutations was used to examine the distribution of genetic variation.

Population genetic structure across all the studied breeds was investigated using a Bayesian approach implemented in STRUCTURE software version 2.3.4 (Pritchard et al., 2000) to estimate ancestral clusters ( $K$ ). The assignment of individuals to populations/breeds, considered an ancestry model with admixture, correlated allele frequencies and defined sampling location for each individual. Ten independent runs with 500,000 MCMC iterations and a burn-in of 300,000 steps were performed for  $1 \leq K \leq 11$  to estimate the most likely number of clusters present in the dataset. The STRUCTURE output was analysed in HARVESTER (Earl and vonHoldt, 2012). The most likely number of clusters was identified by the  $\Delta K$  method (Evanno et al., 2005).

To assess the degree by which populations/breeds differ from each other when adopting an approach without the HWE or LD assumptions, a Discriminant Analysis of Principal Components (DAPC) was performed using the ADEGENET package approach (Jombart, 2008) and statistical package R version 4.0.3. DAPC was carried out without a posteriori group assignments by inferring the most likely number of genetic clusters ( $K$ ) using the find clusters function in the ADEGENET package. The utility  $K$ -means clustering was used to calculate a Bayesian information criterion (BIC) value for each probable value of  $K$  (the most likely  $K$  has the lowest BIC value) and describes individual group assignments for DAPC.

## 2.3. Mitochondrial DNA analysis

A total of 223 mtDNA control region sequences belonging to the eight Mediterranean dog populations/breeds were analysed (Supplementary Material S1). The D-loop region between sites 15,406 and

16,117 was amplified using the following primers: forward 5'-CTTGCTCCACCATCAGCACC-3' and reverse 5'-TGTCCTGAAACCATTGACTGA-3', retrieved from Imes et al. (2012) and Kowalczyk et al. (2015), respectively, and based on the canine reference sequence reported in GenBank with the accession number NC002008 (derived from U96639).

Amplification reactions were performed on 10–30 ng of template DNA in 25  $\mu$ l of reaction mix (Nxumalo et al., 2020). The PCR fragment of 712 bp encompassing the entire mtDNA hypervariable region 1 (HV1) was first purified using exonuclease I and alkaline phosphatase (ExoSAP-IT® enzymatic system-USB Corporation, Cleveland, OH, USA), then Sanger-sequenced with the primer forward 15414F (5'- ACCATCAG-CACCCAAAG -3'), specifically designed by using Primer3 software ([www.primer3.ut.ee/](http://www.primer3.ut.ee/)).

Sequences were assembled and aligned to the reference sequence NC002008 using Sequencher™ 5.10 (Gene Codes Corporation, Ann Arbor, MI, USA). Whenever electropherograms showed ambiguities, new PCR amplifications and sequencing reactions were performed. All novel sequences identified were deposited in the GenBank database under accession numbers MZ484967-MZ485189.

Resulting haplotypes were classified following the Canis mtDNA HV1 database (CHD), that can be accessed at <http://chd.vnbiology.com>. It serves as a tool supporting the scientific community in naming new HV1 haplotypes and to reconcile existing annotation of HV1 sequences.

Several mtDNA sequence variation parameters were estimated by using DnaSP 5.1 software (Librado and Rozas, 2009). Analysis of MOlecular VAriance (AMOVA) and pairwise  $F_{ST}$  calculations were performed using the Arlequin 3.5.2 software package (Excoffier and Lischer, 2010).

The evolutionary relationships among haplotypes were visualized through the network construction obtained with the Network 4.6 software (<https://www.fluxus-engineering.com/>) using the median-joining algorithm followed by the maximum parsimony option for the complexity reduction. All characters were unweighted (all 10), except for mutations at nucleotide positions (nps) 15,612 and 15,652 that were weighted at 30 due to their redundancy.

Principal component analysis (PCA) was performed using Excel software implemented by XLSTAT (<https://www.xlstat.com/>), by considering haplotype frequencies as source data (as previously performed in Cardinali et al., 2016). Standardized Cronbach's alpha value was estimated to assess the reliability of the principal component analysis (Cronbach's alpha below 0.6 was not accepted).

Finally, after trimming the sequences to fit within the same range (140 bp from the HVSI), hierarchical clustering was conducted in MEGA7 (Kumar et al., 2016) by using the UPGMA algorithm and including the only single Kelb tal-Fenek mtDNA sequence available in GenBank (mitochondrial control region sequence with accession number EU223665.1) and 56 European ancient DNA sequences retrieved from Dryad database that are limited to the HVSI (Ollivier et al., 2018).

## 3. Results

### 3.1. Microsatellite diversity

A mean of almost 11 alleles were detected among the 16 loci investigated, and following the Benjamini and Hochberg correction, only AHTh260 was noted to have deviation from HWE in just two of the populations/breeds. INRA21 exhibited the lowest number of alleles (7), whereas the maximum value belongs to AHT137 (18). The values of expected heterozygotes ranged from 0.50 (REN247M23) to 0.78 (AHTh260), while the observed span from 0.49 (REN247M23) to 0.78 (REN169O18) (Supplementary Table S2). The average microsatellite marker PIC value was 0.65. Only REN247M23 locus, had a PIC value smaller than 0.50, and among these values, six markers showed PIC values higher than 0.70.

The mean number of observed alleles, allelic richness per

population/breed, mean observed and expected heterozygosity, and inbreeding coefficient per population/breed are presented in Table 1. MNA values ranged from the lowest in the Kelb tal-Fenek breed ( $4.44 \pm 1.63$ , same as German Shepherd), to the maximum value in the Egyptian Baladi street dog population ( $8.31 \pm 1.92$ ). In line with this, the parameters of variability (Rt, Ho and He) for the Egyptian Baladi street dog population appeared as boundary values for the highest values ( $4.94; 0.77 \pm 0.02; 0.79 \pm 0.02$  respectively), and in the case of the Kelb tal-Fenek for the lowest ones ( $3.15; 0.56 \pm 0.02; 0.58 \pm 0.05$  respectively). On the contrary, the  $F_{IS}$  values for inbreeding evaluation highlighted different results: Podenco Canario has the lowest values ( $-0.02957$ ), whereas Cirneco dell’Etna the highest inbreeding value ( $0.07050$ ). Nonetheless, a low level of inbreeding was noted in all the studied populations/breeds.

To describe the level of heterogeneity within and between the selected Mediterranean dog populations/breeds, *F*-statistics values and AMOVA analysis were performed (Supplementary Table S3). Results of variance attributed 8.71% due to variations among population, while a major part of variance was due to variations within individuals. Moreover, genetic variation among groups ( $F_{IS}$ ) and genetic variation among populations within groups ( $F_{IT}$ ) showed significant positive values ( $0.004$  and  $0.087$ , respectively;  $P < 0.01$ ).

The constructed Neighbor-Net tree of the dog populations in this study is presented in Fig. 1. The German Shepherd breed, as expected, placed very distant from the study groups. It is worthy to note that in this study, the Kelb tal-Fenek breed that geographically lives in the centre of the Mediterranean, does not reveal to have strong connections with the other populations/breeds. However, this finding should be verified with further studies and including other breeds, geographically and phenotypically close to Kelb tal-Fenek, that were not considered in this study to explore the influences on its genetic background. These results are also supported by the pairwise  $F_{ST}$  distances shown in Table 2. Indeed, the Kelb tal-Fenek population has the highest values ( $F_{ST}$  ranging from  $0.104$  to  $0.176$ ), in line with what is reported in Fig. 1. The most similar populations/breeds seem to be the two Podenco breeds from the Spanish mainland (Podenco Andaluz and PodencoValenciano) and the Egyptian Baladi street dog. Indeed, pairwise genetic differentiations quantified by  $F_{ST}$  estimates singled out Egyptian Baladi street dog and Podenco Andaluz ( $0.012$ ) as being the closest populations/breeds although with a not significant level, as well as the proximity between Egyptian Baladi street dog and Podenco Valenciano ( $0.025$ ), and between Podenco Andaluz and Podenco Valenciano ( $0.016$ ) (Table 2). A possible explanation could be an extensive gene flow between breeds/populations that might have contributed, at least in part, to the genetic makeup of these breeds. This situation could be also explained, in part, by the low resolution due to type of genetic markers used. The Podengo Português merits a separate mention, since while it did not place so close to these three breeds as depicted in Fig. 1, it showed a close  $F_{ST}$  distance with

Podenco Valenciano, Podenco Andaluz, and Egyptian Baladi street dog ( $0.045$ ,  $0.032$ , and  $0.041$  respectively) (Table 2). Furthermore, all the island populations/breeds (Canarias, Sicily, Malta, and Ibiza) seem to have had a degree of genetic isolation that resulted in distant relations with other populations/breeds (Fig. 1). In fact Table 2 shows that the highest  $F_{ST}$  distance values are between populations/breeds from island (Podenco Canario, Cirneco dell’Etna, Kelb tal-Fenek, and Podenco Ibicenco), with the maximum values between for Kelb tal-Fenek and Podenco Ibicenco ( $0.169$ ).

The genetic relationship between populations/breeds is further validated by the Discriminant Analysis of Principal Components (DAPC). The Bayesian Information Criterion (BIC) statistic showed that the optimal number of clusters in the data set is  $K = 7$  (Supplementary Fig. S1). In the DAPC analysis, 70 PCs of the PCA were retained as input to discriminant analysis, accounting for approximately 93% of the total genetic variability. The scatterplot of the first three components of the DA highlighted extensive sharing of genetic variation among Egyptian Baladi street dog, Podengo Português, Podenco Andaluz, Podenco Ibicenco, Podenco Valenciano and Cirneco dell’Etna, which formed a tight cluster with no visible discernible structure. Although 95% confidence ellipses for the Kelb tal-Fenek and Cirneco dell’Etna did not overlap, they still appear in close proximity to each other (Fig. 2).

Admixture analyses were performed defining seven ancestral populations (*K*). The STRUCTURE software was used to evaluate and compare the genetic structure of the nine populations including the German Shepherd breed as an outgroup, and  $K = 7$  was chosen as the most representative. Podenco Ibicenco, Podenco Canario, and especially Kelb tal-Fenek populations/breeds show a defined and unique structure. The same should be said for Cirneco dell’Etna that appear as a unique subpopulation with some admixtures, and similarly in Podenco populations/breeds and Egyptian Baladi street dog too (Fig. 3). However, these results need to be carefully evaluated as the evidence of admixture could be in fact be due to either a low resolution of markers or to the presence of a shared ancestry.

### 3.2. Mitochondrial DNA diversity

The molecular analysis of 622 base pairs of the mitochondrial HV1 region (from np 15,461 to np 16,082) on the entire dataset revealed a total of 36 distinct haplotypes (Supplementary Table S4). Nucleotide diversity ( $\pi$ ) across all populations/breeds was estimated at  $0.012$ ; the haplotype diversity ( $H_d$ ) at  $0.879$ . The highest haplotype diversity was detected in the Egyptian Baladi street dog ( $H_d = 0.945$ ), followed by the Podenco Andaluz ( $H_d = 0.903$ ). The lowest value ( $H_d = 0.692$ ) was registered in the Podenco Ibicenco breed.

Although 32 Kelb tal-Fenek samples were analysed in this study, only four haplotypes were detected, resulting in a low overall haplotype diversity ( $H_d = 0.744$ ).

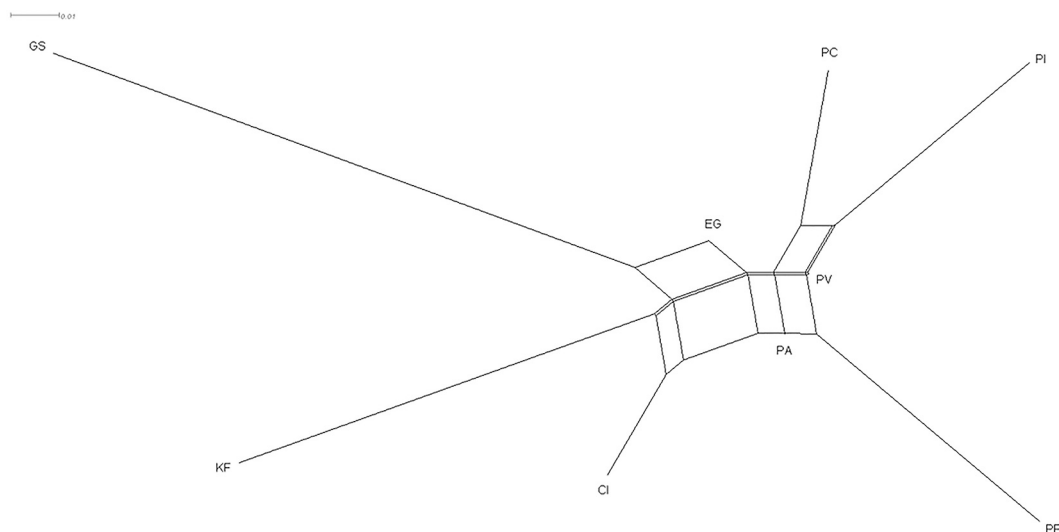
**Table 1**

Dog breeds, sample size of each breed (N), mean number of observed alleles (MNA), allelic richness per breed (Rt), mean observed (Ho) and expected heterozygosity (He), and inbreeding coefficient ( $F_{IS}$ ) per breed/population.

Breed/population	N	MNA $\pm$ SD	Rt*	Ho $\pm$ SD	He $\pm$ SD	$F_{IS}$ [IC 95%]
PV	61	7.88 $\pm$ 2.60	4.59	0.71 $\pm$ 0.01	0.76 $\pm$ 0.02	0.06697 [0.02365–0.09291]
PI	38	5.56 $\pm$ 1.67	3.36	0.68 $\pm$ 0.02	0.67 $\pm$ 0.02	–0.01014 [–0.07338–0.02499]
PC	30	6.44 $\pm$ 1.55	4.05	0.70 $\pm$ 0.02	0.68 $\pm$ 0.04	–0.02957 [–0.11069–0.00966]
PA	31	7.31 $\pm$ 2.15	4.58	0.73 $\pm$ 0.02	0.76 $\pm$ 0.02	0.04522 [–0.02831–0.08232]
PP	17	5.56 $\pm$ 1.41	4.04	0.68 $\pm$ 0.03	0.70 $\pm$ 0.03	0.02098 [–0.13543–0.07844]
KF	32	4.44 $\pm$ 1.63	3.15	0.56 $\pm$ 0.02	0.58 $\pm$ 0.05	0.04105 [–0.04112–0.07730]
EG	30	8.31 $\pm$ 1.92	4.94	0.77 $\pm$ 0.02	0.79 $\pm$ 0.02	0.01951 [–0.04386–0.04359]
CI	33	7.56 $\pm$ 2.22	4.50	0.70 $\pm$ 0.02	0.76 $\pm$ 0.02	0.07050 [–0.01595–0.12434]
<b>Mean</b>		<b>6.63 <math>\pm</math> 1.89</b>	<b>4.15</b>	<b>0.69 <math>\pm</math> 0.02</b>	<b>0.71 <math>\pm</math> 0.03</b>	<b>0.028065 [–0.01231–0.07918]</b>
GS	29	4.44 $\pm$ 1.36	3.11	0.63 $\pm$ 0.02	0.60 $\pm$ 0.03	–0.04857 [–0.13853–0.00515]

PV, Podenco Valenciano; PI, Podenco Ibicenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Português; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell’Etna; GS, German Shepherd.

\* : based on 5 individuals.



**Fig. 1.** NeighborNet graph based on Reynolds genetic distances for the nine dog populations/breeds.

PV, Podenco Valenciano; PI, Podenco Ibicenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Português; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell’Etna; GS, German Shepherd.

**Table 2**

Pairwise  $F_{ST}$  distance between the nine dog breeds/populations studied.

	PV	PI	PC	PA	PP	KF	EG	CI	GS
PV	0								
PI	0.042	0							
PC	0.048	0.092	0						
PA	0.016	0.073	0.042	0					
PP	0.045	0.108	0.095	0.032	0				
KF	0.104	0.169	0.109	0.116	0.176	0			
EG	0.025	0.088	0.044	0.012 <sup>ns</sup>	0.041	0.104	0		
CI	0.062	0.141	0.104	0.064	0.067	0.123	0.063	0	
GS	0.121	0.187	0.145	0.117	0.165	0.148	0.090	0.138	0

ns: not significant;  $P < 0.05$ .

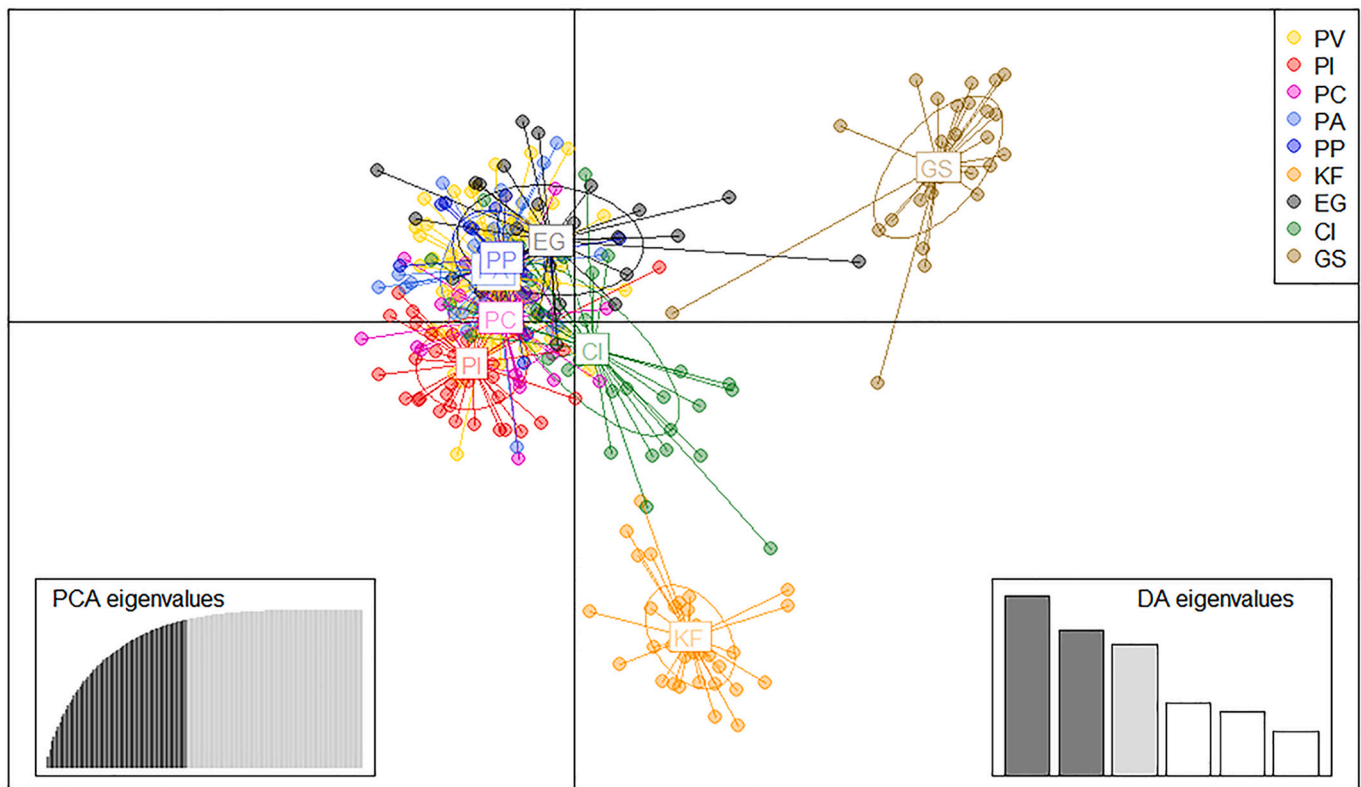
PV, Podenco Valenciano; PI, Podenco Ibicenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Português; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell’Etna; GS, German Shepherd.

The maternal genetic sub-structure was also assessed within and between the studied populations/breeds by analysis of sequence variations (AMOVA). The molecular variance analysis established that almost all the observed variance is attributable to differences within populations (98.54%), while only the remaining 1.46% of the genetic variation being attributed to the among-populations/breeds’ component associated with a value of the fixation index ( $F_{ST} = 0.01455$ ) (Supplementary Table S5).

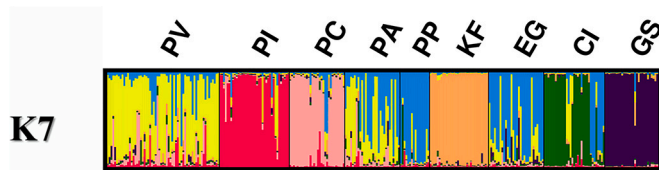
The resulting median-joining network of the control regions exposed a few points of reticulations caused by recurrent mutations that not altered the network topology (Fig. 4). It clearly defined four major branches that correspond to the canine haplogroups that have been identified so far (Duleba et al., 2015; Yankova et al., 2019). Haplotype and haplogroup classifications were confirmed through an accurate analysis of diagnostic mutational motifs identified among the control-region haplotypes and refined with the Haplotype Identifier Tool of the CHD (Thai et al., 2017). Each mtDNA sequence was used as an entry in CHD database to identify the haplotype. If the mutation profile was identical to an already assigned haplotype, the haplotype was defined accordingly, thus named by the corresponding haplogroup followed by the same Arabic numeral. This tool recognised a total of 220 sequences into 33 known haplotypes. Three entries did not match with assigned haplotypes and were thus considered as being candidates for new haplotypes. These were classified as the “Nearest haplotype” and reported in the “Haplotype Identifier” in CHD, thus avoiding additional haplotype numbering (Supplementary Material S1).

According to this system, all mtDNA haplotypes belong to four phylogenetic groups labelled A to D, with high prevalence of haplogroup A (55.2%) and B (40.8%). Haplogroup A consists in three prevailing haplotypes (A11, A18, and A20) and sub-branches with several private haplotypes. Haplotypes related to haplogroup B showed a star-like pattern around the most frequent haplotype (B1) and the difference between haplotypes in this haplogroup is mostly a single nucleotide. Haplogroup C and D were down-represented (3.6% and 0.4%, respectively) and no sequences belong to E and F haplogroups. As evident in Fig. 5, four populations/breeds (Cirneco dell’Etna, Kelb tal-Fenek, Podenco Ibicenco, and Podenco Canario) showed only the A and B haplogroups, with an almost homogenous frequency. The Podenco Ibicenco had exactly 50% of A Hg and 50% of B Hg. The rest of the populations/breeds (Egyptian Baladi street dog, Podenco Andaluz, Podengo Português, and Podenco Valenciano) also revealed the presence of C haplogroup with only one haplotype per population/breed: Egyptian Baladi street dog and Podengo Português shared one haplotype, while Podenco Andaluz and Podenco Valenciano both showed unique haplotypes (Fig. 6). The Egyptian Baladi street dog was the only population with a D haplogroup, and it was detected in only one sample.

The stochastic distribution of our haplotypes among haplogroups confirms that it is not possible to identify population/breed-specific mitochondrial clades, at least at this level of resolution as previously reported in dogs (Smalling et al., 2010) and also in other mammals such as sheep (Lancioni et al., 2013), cattle (Di Lorenzo et al., 2016) and horse breeds (Cardinali et al., 2016; Lancioni et al., 2020; Engel et al., 2021).



**Fig. 2.** Scatterplot of the first three principal components of DAPC using breeds as a posteriori cluster. PV, Podenco Valenciano; PI, Podenco Ibicenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Portugués; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell’Etna; GS, German Shepherd.



**Fig. 3.** Clustering of nine dog populations/breeds with STRUCTURE. PV, Podenco Valenciano; PI, Podenco Ibicenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Portugués; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell’Etna; GS, German Shepherd.

With reference to the Kelb tal-Fenek, sequencing results revealed only four haplotypes (A18, A20, B1, and B10) corresponding to the most frequent haplotype (HT) in all the dataset (B1 = 26%, A20 = 15%, A18 = 10%, and B10 = 7%). In addition, B1 is the only HT found in all the tested populations/breeds, followed by A18 that was found in all populations/breeds except for Podenco Ibicenco. A20 was shared with all populations/breeds except the Cirneco dell’Etna and Podengo Portugués, while B10 was shared by Egyptian Baladi street dog, Podenco Andaluz, Podenco Ibicenco, and Kelb tal-Fenek. The schematic representation of the phylogenetic tree (Fig. 6), consistent with the genetic distances between and within populations/breeds showed in Supplementary Fig. S2 and Table 3 describe Kelb tal-Fenek as being most genetically related to Podenco Ibicenco, followed by Podenco Andaluz and Podenco Valenciano. While the Podengo Portugués and Egyptian Baladi street dog are confirmed to be the most distant populations/breeds.

The principal component analysis (PCA) shown in Supplementary Fig. S3 graphically displays the outlier position of Egyptian Baladi street dog, particularly along the first principal component that clearly splits all other populations/breeds in the second and third quarter of the

graph.

#### 4. Discussion

##### 4.1. Genetic structures within populations/breeds

The populations/breeds studied belong to the main mitochondrial haplogroups A, B and C, which exhibit a wide old-world distribution (Savolainen et al., 2002). Only one Egyptian Baladi street dog sample behaved as an outlier and assigned with haplogroup D. The overall frequencies of Hg A, B, and C (55.2%, 40.8%, and 3.6% respectively) are similar (58.3%, 41.3%, and 0.4%, respectively), to those reported by Suárez et al. (2013) for different populations/breeds within the micro-geographic context of the Canary Islands. Even if these proportions are similar to those obtained in other studies, the haplogroup B shows considerably higher frequency when compared to the observed frequency of approximately 20% in Europe by Savolainen et al. (2002) and Duleba et al. (2015). A recent study focusing on the genetic diversity of local Balkan hunting dogs reported the same distributions to be found in the Bulgarian Hound dogs (53% for Hg A, and 24% for both B and C) (Marinov et al., 2018). In our data only the Egyptian Baladi street dog and the Podengo Portugués registered <30% of the Hg B frequency, while all the other population/breed recorded over 35% with a peak of 55% found in the Podenco Valenciano. Even if this over-representation of Hg B in our data could be due to the small breed consistency and/or due to sample selection bias, it could still enforce the statement of Yankova et al. (2019) when argued that the spreading of this clade across Europe, is related to the possible dissemination of newly formed dog breeds from Ancient Greece, Thrace, and the Roman Empire eras.

Genetic diversity was evaluated through the level of inbreeding ( $F_{IS}$ ) and the observed and expected heterozygosities ( $H_O$  and  $H_E$ , respectively) for the studied populations/breeds (Table 1). The heterozygosity

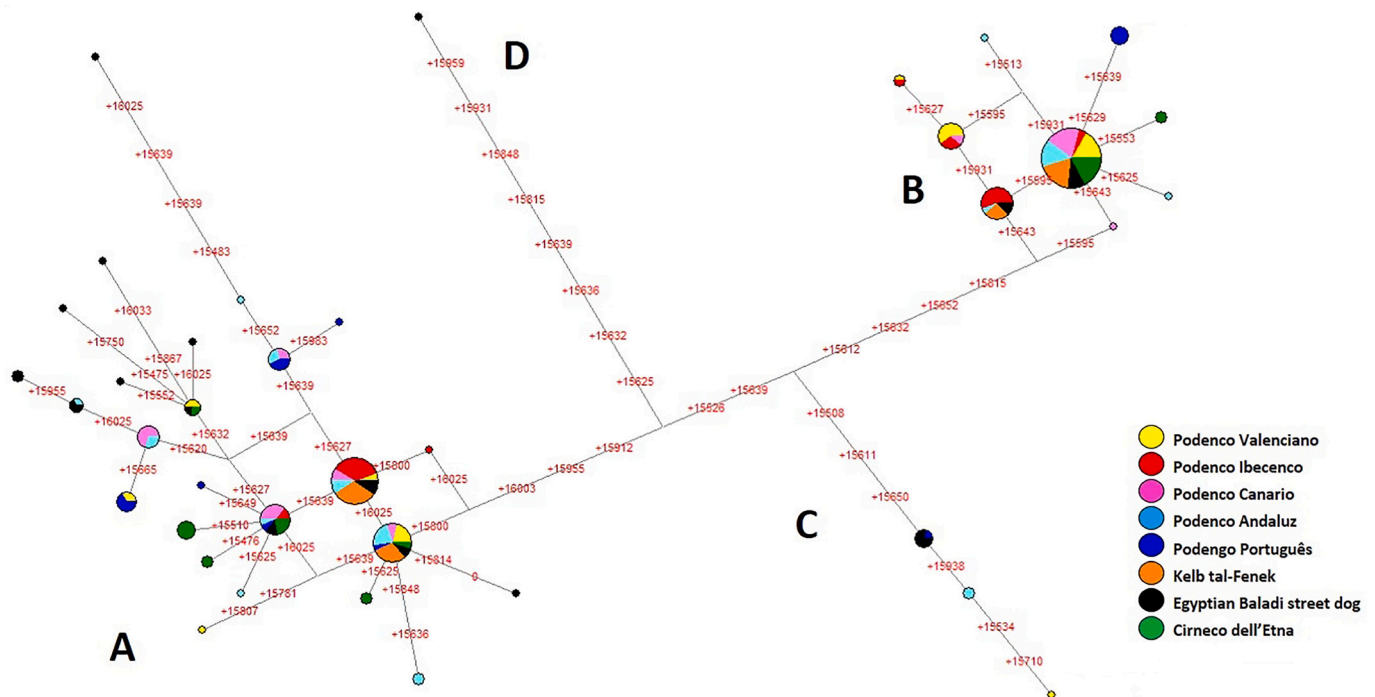


Fig. 4. Median-Joining Network based on mitochondrial HV1sequences.

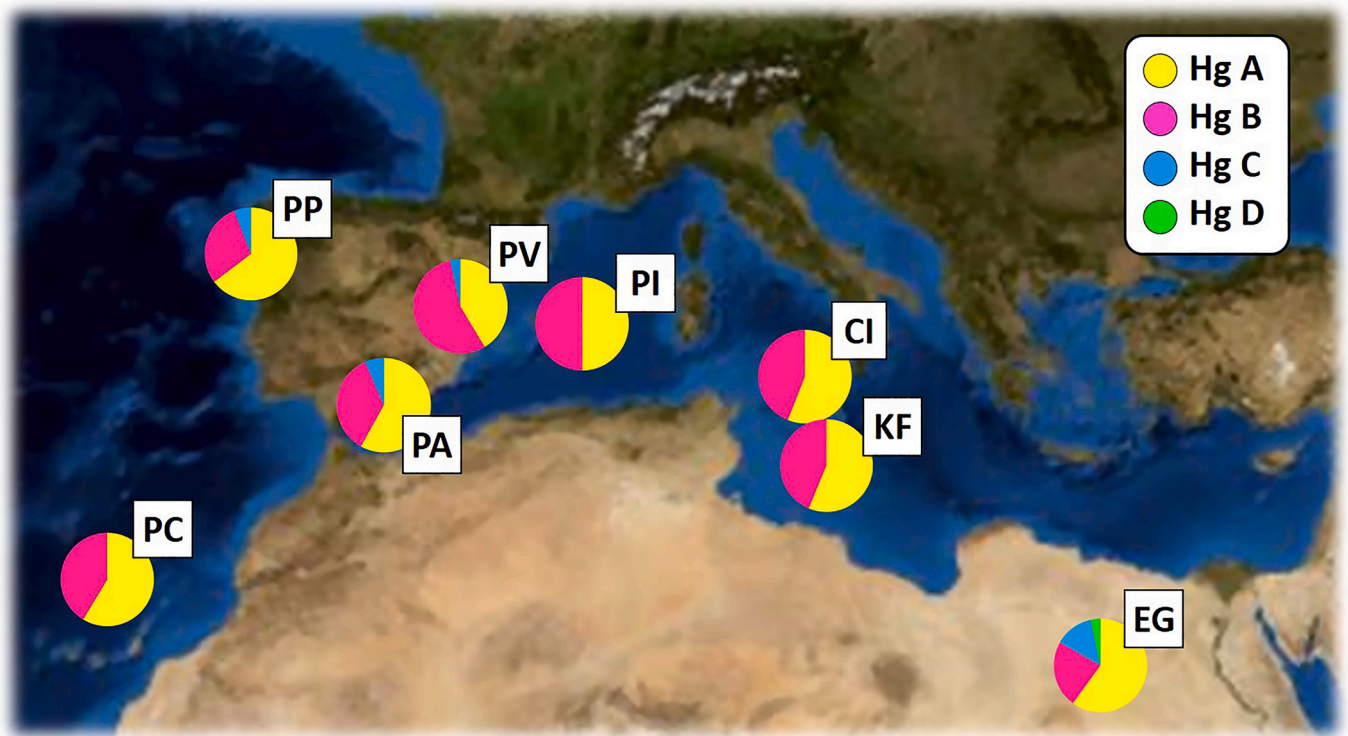
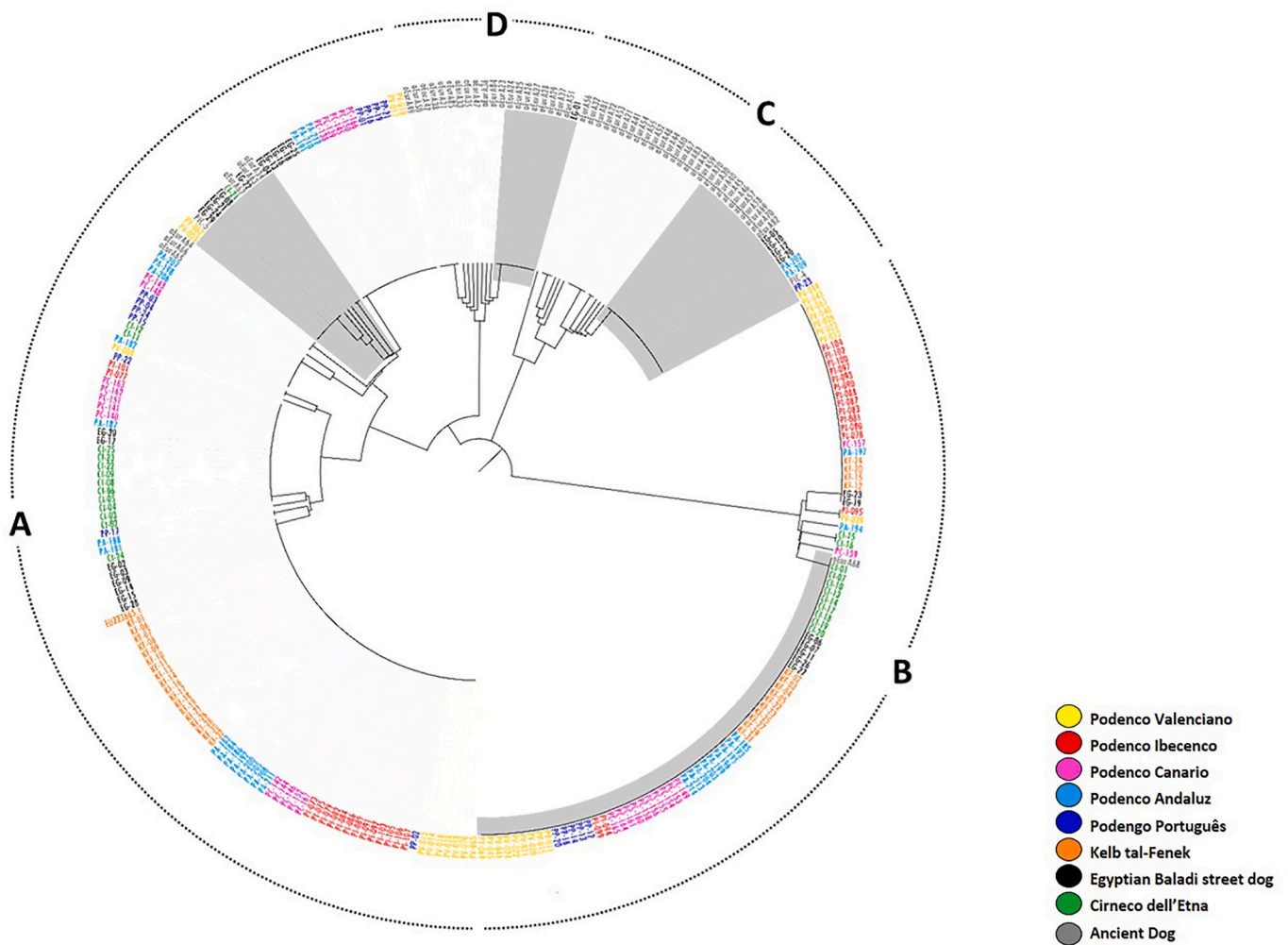


Fig. 5. Frequency distributions of mitochondrial haplogroups. USGS National Map Viewer (public domain): <http://viewer.nationalmap.gov/viewer/> PV, Podenco Valenciano; PI, Podenco Ibecenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Portugués; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell'Etna; GS, German Shepherd.

values ( $H_e$  mean = 0.71) are higher than what was previously reported in similar studies. (Irion et al., 2005; Bigi et al., 2015). At the same time, the level of heterozygosity recorded in indigenous dog populations/breeds are in line with our results, as reported for Italian dog breeds ( $H_e$  from 0.62 to 0.77) (Bigi et al., 2015) and for the Yugoslavian shepherd

dog ( $H_e = 0.70$ ) (Dimitrijevic et al., 2013). The observed high levels of heterozygosity can be the result of many factors, such as the low values of inbreeding, less artificial selection and therefore a higher level of random mating and the possibility of gene flow that can occurred in local breeds when compared to the modern breeds. Moreover,



**Fig. 6.** Schematic phylogenetic tree of modern and ancient mitochondrial HV1 sequences. The terminal branches shared between ancient and modern mtDNAs are shaded.

**Table 3**  
Population average pairwise differences.

	PV	PI	PC	PA	PP	KF	EG	CI
PV	7.3966	6.9598	7.5386	7.7152	8.3286	7.0571	8.2081	7.6441
PI	0.0316	6.4598	6.9931	7.1731	7.7255	6.4125	7.6633	7.1613
PC	0.1753	0.0982	7.3301	7.4004	7.5538	6.9537	7.7230	7.3766
PA	0.1460	0.0723	-0.1356	7.7419	7.8691	7.0434	7.8409	7.6645
PP	0.6156	0.4809	-0.1260	-0.0166	8.0294	7.6746	7.9863	8.0471
KF	0.1098	-0.0664	0.0397	-0.0766	0.4109	6.4980	7.5813	7.1088
EG	0.4730	0.3967	0.0212	-0.0669	-0.0652	0.2955	8.0736	8.0627
CI	0.1142	0.0998	-0.1201	-0.0381	0.2007	0.0281	0.1942	7.6633

Above diagonal: average number of pairwise differences between populations; diagonal elements: average number of pairwise differences within population; below diagonal: corrected average pairwise difference.

PV, Podenco Valenciano; PI, Podenco Ibecenco; PC, Podenco Canario; PA, Podenco Andaluz; PP, Podengo Portugués; KF, Kelb tal-Fenek; EG, Egyptian Baladi street dog; CI, Cirneco dell'Etna.

opportunities for outbreeding may have occurred since there may have been less stringent human imposed boundaries. Most of the modern dog breeds can be described as being closed breeding populations, in which artificial selection mainly focuses on conformation traits. Indeed, Pedersen et al. (2013) support the idea that selection for morphological traits leads to high inbreeding, while selection for performance tends to maintain biodiversity. In fact, apart from the Egyptian Baladi street dog, all the selected populations/breeds in the present dataset were originally selected to promote and enhance their hunting abilities and not

according to conformation traits as is required by the stricter pedigree of cosmopolitan breeds.

On average, there was no significant deficit of heterozygotes ( $F_{IS} = 0.028$ ); the value being lower than that reported by Ciampolini et al. (2011) ( $F_{IS} = 0.061$ ), by Morera et al. (1999) ( $F_{IS} = 0.085$ ). The two Spanish Podencos (Valenciano and Andaluz) showed  $F_{IS}$  values in line with a previous study (Jordana et al., 1992). The lowest values for  $F_{IS}$  attributed to Podenco Ibecenco and Podenco Canario from two small islands are not in agreement with other studies; Suárez et al. (2013)



reported a higher and significant level for  $F_{IS}$  in the Podenco Canario breed, and Jordana et al. (1992) found similar value for the Podenco Ibicenco breed.

#### 4.2. Genetic differentiation and genetic affinities among populations/breeds

The among populations/breeds genetic variation in this study stands at around 8.71%. Pairwise  $F_{ST}$  distances for the Spanish Podenco were reported also by San José et al. (2018) and are in line with data from this study, thus underlying, as expected, the closeness of the populations/breeds, especially between Podenco Valenciano and Podenco Andaluz (0.016 present study; 0.018 San José et al., 2018). Based on the  $F_{ST}$  results of microsatellites, the Kelb tal-Fenek seems to be a distinct population from the current Egyptian Baladi street dog, but an ancient relationship cannot be excluded, and additional studies are required to further investigate this aspect.

The Kelb tal-Fenek registered the highest divergences among all the other populations/breeds included in this study. This is consistent with the STRUCTURE analysis (Fig. 3) in which the Kelb tal-Fenek shows a defined structure as in Fig. 1 where it is the most distant breed among the study populations. It is a popular opinion that due to commercial routes in the Mediterranean Sea, the Cirneco dell'Etna, Kelb tal-Fenek, and the Podenco breeds had a connection in the past, and hence a genetic connection was highly probable. Moreover, in 1647 Abela gives the first reference to the existence of a local type of dog in Malta, highly esteemed for its rabbit hunting abilities over harsh and rocky terrain and which he claims to resemble the Cirneco dell'Etna. Hence although a common ancestral history between the Cirneco dell'Etna and Kelb tal-Fenek was assumed, our results indicate a wider genetic distance. On the other hand, Cortellari et al. (2021) reported that the Kelb tal-Fenek and Cirneco dell'Etna seem to have relatively close connection from a genetic point of view. Our results highlight that contrary to popular belief, the Cirneco dell'Etna seems to have a much stronger connection with Podenco Valenciano, Podenco Andaluz, Podengo Português, and Egyptian Baladi street dog, than with the Kelb tal-Fenek.

#### 4.3. Phylogeographic structuring of dog local populations/breeds in the Mediterranean Basin

The only data available that is specifically linked with typical Mediterranean local populations/breeds is found on one control region on the mtDNA belonging to a Kelb tal-Fenek (EU223665) and 22 sequences from the Egyptian Baladi street dog (MK050465-MK050486). While all these sequences were recorded in GenBank, the limited number of available sequences undoubtedly represents a constraint in this study. The Kelb tal-Fenek sample (EU223665) was included in a publication (Webb and Allard, 2009) and compared to the mtDNA of other dog breeds in a forensic investigation. It consists of a very common haplotype (A20), found in 11 out of the 32 Kelb tal-Fenek and also in all of the Egyptian Baladi street dog and the Podenco breeds except for the Podengo Português. Webb and Allard (2009) reported the same HT for a variety of very different breeds (Chihuahua, Coton de Tulear, Maremma, Papillon/Sheltie, and Pointer). Haplotype A20 is reported by Suárez et al. (2013) as being the most frequent in Europe, together with A11. The haplotype A11 was identified in all the selected dog populations/breeds but not in the Kelb tal-Fenek and Podenco Valenciano breeds. This A11 haplotype is largely distributed across Southwest Asia and East Asia (Amiri Ghanatsaman et al., 2018; Ardalan et al., 2011; Pang et al., 2009). Verscheure et al. (2013) classifies A11 as one of the three most frequent haplotypes (together with B1 and A17). A recent study has for the first time shown the presence since the Neolithic Period of clade B in ancient Eurasia, with a peak of 40% attributed to the Chalcolithic era (Yankova et al., 2019). In a previous study conducted on ancient dogs and wolves from southern Europe, Verginelli et al. (2005) also revealed Hg B in wolves. The presence of this clade in both extant wolves and

dogs was explained with hybridization events even before the Neolithic period followed by dispersal across Europe (Yankova et al., 2019).

Thanking into consideration all available ancient dog mtDNAs, there is a general consensus among researchers that clade A is associated with the Neolithic farmer migration from the Middle East into ancient Europe, while clade B probably originated independently from South-Eastern Europe. However, the overall data from pre-historic Europe shows a dramatic difference between ancient and present-day dog populations, that can be explained with the replacement of local dogs with the more improved breeds mainly from the southern part of Europe, the Mediterranean region, and the Near East, during the Late Antiquity and Medieval period. Concurrently, the extensive turnover of mtDNA haplotypes between ancient and modern dogs led to the inversion of the haplogroup distribution, during which clade A rose to a higher frequency while clades B, C, and D decreased (Koblmüller et al., 2016; Verginelli et al., 2005; Yankova et al., 2019).

The sequences from this study were compared with the only Kelb tal-Fenek mtDNA sequence available in GenBank (EU223665) and with ancient European dog mtDNAs retrieved from the Dryad Digital Repository package (Supplementary Material S2), by building a schematic phylogenetic tree encompassing modern (224) and ancient (56) mitogenomes (Fig. 6). All the four main clades revealed terminal branches that are shared between ancient and modern mtDNAs. Data from ancient dog is overwhelmingly represented in Hg C and D, while seven ancient dog sequences fall in Hg A and only one in Hg B. The latter was collected in South France, while, within Hg A, our EG-04 sample (Egyptian Baladi street dog) is identical to a sample from East France and shows close similarities to other sequences from Russia. As expected, when considering all the published data on the shared haplotypes that the hound-like native dogs share with many other breeds, the results of the Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) verified that at this level of resolution it is not possible to identify population/breed-specific mitochondrial lineages. Finally, the Italian canid PIC-5 dated to the recent Bronze Age showed haplotype identity with Cirneco dell'Etna, Podenco Valenciano and Egyptian Baladi street dog sequences obtained in this study. While most modern European dogs harbour haplogroups A and B (64 and 22%, respectively), clade C, together with D, were predominant in ancient European dogs that lived some 7000 years ago (63 and 20%, respectively), but were not prevalent in the Middle East, where the frequencies were considerably lower (Frantz et al., 2016). Within the Hg C, the shared clade included the Egyptian Baladi street dog, Podenco Valenciano, Podengo Português, Podenco Andaluz and 11 ancient dog sequences originating from Italy, Switzerland, France, Germany, and Romania. This suggests the conservation over several millennia of ancient European mitotypes in the Mediterranean native houndlike populations/breeds. To this effect, the Italian canid PIC-4 was attributed to the Eneolithic era. Interestingly, both prehistoric Italian canids that are attributed to medium-sized dogs (Verginelli et al., 2005), revealed genetic continuity with the Podenco Valenciano and Egyptian Baladi street dog suggesting an ancient origin of these two populations/breeds. A very recent study conducted in South Italy unveiled the oldest evidence of dogs in the Mediterranean and indicated how this Upper Palaeolithic dog C lineage may have contributed to the genetic diversity of living dogs (Boschin et al., 2020).

Finally, the tree in Fig. 6 also revealed the presence of haplotype identity within clade D between the Egyptian Baladi street dog and eight ancient dog mtDNAs from Romania and Moldova. Overall, the Egyptian Baladi street dog population is the one that better describes the genetic continuity with the ancient samples. Considering all the available sequences of the Egyptian Baladi street dog (MK050465-MK050486) for a total of 52 mtDNA sequences, a very high level of haplotype diversity ( $H_d = 0.955$ ) was noted. This is not surprising because it is a stray semi feral dog that lives on the streets and rural countryside, now mixed with other popular populations/breeds, so that even the definition of breed is questionable.

According to the numerous archaeological remains and

representations in engravings, statues and bas-reliefs dated back to over 7000 years before present, scholars suggest that the Podenco Canario is one of the oldest breeds known today. It is considered to be native of Egypt, and was probably taken by the Phoenicians, Greeks and Carthaginians or the same Egyptians to the Canary Islands. Also, the Kelb tal-Fenek is sometimes claimed to have descend from the dogs shown in tomb paintings of Ancient Egypt, as the English-language exonym suggests. A recent comparison of Kelb tal-Fenek and Cirneco dell'Etna whole-genome SNP data indicates that Kelb tal-Fenek from the island of Malta and the Podenco Ibicenco from the island of Ibiza are the closest genetic relatives to the Cirneco dell'Etna, but even if the genetic foundations of these breeds date to the more distant past, their separate breed formation occurred within the last 200 years (Talent et al., 2018).

None of populations/breeds selected in this study can be classed as native to Egypt, yet they still closely resemble the ancient iconic Egyptian dogs, not only for their phenotypic traits, but also as indicated in this study by the potential genetic links as is being suggested by haplotype sharing. Thus, contemporary Podenco hounds still preserve lineages with the ancient dog type, probably, as stated by many non-official records, due to the disseminated of the dog type across the Mediterranean Basin by the Phoenicians during the first millennium BCE. Recent studies focusing on the genetic history of human populations inferred an extensive exchange and continuity between the Phoenicians and other Mediterranean populations (Matisoo-Smith et al., 2018; Fernandes et al., 2020; Marcus et al., 2020), thus supporting the hypothesis that while the Phoenician sailors established settlements in Malta, Sicily, Ibiza, the Iberian Peninsula and also along the North African coast, they also might have brought along dogs (Zalloua et al., 2008).

The authors are aware that the limitations are not only due to the paucity of the dataset but also due to the nature of the mitochondrial control region. The maternally transmitted mitochondrial control region is a very small portion of the mitochondrial genome and as such might only provide a partial reconstruction of the matrilineal genetic history, and hence cannot perfectly coincide with the more accurate story provided by other molecular markers. In recognition of the limitation due to the number of microsatellite makers used and the possible existence of population gene flow, this study nonetheless represents the ever first attempt to better describe the genetic structure of hound-like indigenous dogs found in various locations along the Mediterranean. However further research is necessary to integrate data with complete mitogenomes and SNP panels to unravel the complex history of hound-like Mediterranean dogs.

## 5. Conclusions

The lack of a geographic or breed-related structure could indicate that most of the hound-like breeds derived from the same ancestral stock and retain many of the ancestral maternal lineages and/or that an extensive introgression has occurred in the process of breed formation and development.

It is a known fact that any attempt to infer the evolutionary history of dog populations from genomic data can be complex due to the possibility of admixture that could have occurred over time between indigenous and not-local dogs, and probably even with other wild canid lineages. The lack of clear patterns in the distribution of mitochondrial lineages across the houndlike populations/breeds analysed in this study, corroborates the general idea that the origin of dog populations/breeds is to be considered as a recent event, arising from mixed ancestral stock. Above all, the continental Podenco dogs are the hounds that more closely resemble their ancestors and still preserve ancient maternal lineages.

Even if the extent of the evolution of dogs found in the Mediterranean basin requires a broader set of ancient samples from these areas to be fully understood, it is evident that human-mediated selection and exchanges that are also documented by historical evidence, have largely

influenced the effective gene flow within the hound-like dog populations native of the Mediterranean Basin. To date, phenotypic and genotypic peculiarities can be exploited for pedigree breeding management schemes aimed to accurately assess breed distinctiveness and conservation programs.

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## Declaration of competing interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2023.01.010>.

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