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SHORT COMMUNICATION

Genetic differentiation between *Segugio dell'Appennino* and *Segugio Maremmano* dog breeds assessed by microsatellite markers

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Abstract

The domestic dog (*Canis Familiaris*) was the first species to be tamed and bred to occupy a variety of functions. The *Segugio dell'Appennino* and *Segugio Maremmano* are two Italian breeds used extensively as hunting dogs. Microsatellites and other genetic markers are useful tools in evaluating the within and between breed genetic variation. The aim of this study was to investigate genetic differentiation among the two breeds through the analysis of 21 microsatellite markers. The two populations share the same geographical regions but have been over time selected on different parameters to achieve specific functions. All the analysed microsatellite markers were polymorphic and the average number of alleles per locus was 8.19. The mean F_{IT} index (0.051; $P < 0.05$) highlights that at some point in time, the normal gene flow among the animals was disrupted, giving rise to a heterozygote deficiency in both breeds, and this is confirmed by the mean F_{ST} fixation index (0.010; $P < 0.05$) clearly indicating an absence of a significant genetic differentiation between the two breeds. The mean F_{IS} value was significantly different from zero (0.042) ($P < 0.05$) reconfirming the presence of a lack of heterozygosity in the studied samples. The values of observed and expected heterozygosity were similar in the two breeds. AMOVA, PCA and

STRUCTURE analysis, all emphasise the lack of significant differences among the two breeds in terms of genetic differentiation. The presence of a population substructure is probably due to a genetic introgression from different *Segugio* breeds, that can be confirmed with further studies.

Introduction

The domestic dog (*Canis Familiaris*) was the first species to be tamed and has over time been utilised for a vast array of purposes, from a valuable companion to a service animal, and hence selection has generated more phenotypic diversities than in any other mammalian species (Wayne and Vilà, 2001). Moreover, the wide range in size and conformation among the present day dog breeds seems to exceed that found within the species classified in the *Canidae* family (Wayne, 1986).

In Italy, the Italian Kennel Club (ENCI) is the officially recognised entity that defines the breed specification of purebred/pedigree domestic dogs. The *Segugio dell'Appennino* (SA) and *Segugio Maremmano* (SM) are two types of Italian dogs classified under the ENCI group 6, the class that groups the scent hounds and related breeds.

The *Segugio dell'Appennino* has been widely documented and described in popular press that deal with hunting or with canine genetics and breeding, and also in periodicals on living in the Italian countryside and mountainous regions. This breed is said to be of medium-size with stiff short hair, specialised in sniffing out and chasing hares in harsh terrain and in the Appennino mountains. It is noted for its sociable temperament, loyalty to its master, and its striking fast response and speed of action. In the publication *La caccia*, dated 2 November 1882, it was already described morphologically and was grouped among the Italian breeds of scent hounds. Environmental and anthropic selection contributed in the development of this breed which is uniform in morphology, resistant and elegant. In 1932 the lawyer, gentleman farmer and hunter, Filippo Zacchini wrote: *Small-sized hound of great agility and vivacity, all muscles, and nerves are without any heaviness, the origin is very ancient*. Traditional hare game hunters are credited for their diligence in preserving this breed, it was the dog of choice of the small landowners, who provided the dogs with adequate care and close proximity, which in a way also contributed positively to its preservation as a breed in its original appearance. The stan-

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Key words: Italian dog breeds; STR markers; Genetic diversity; *Segugio*.

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dard for the breed was defined and established by ENCI in 2005. The *Segugio Maremmano* originated in the region of Tuscany and most likely was selected and developed morphological characteristics as an established breed towards the end of 1800 as a scent hound to trail large game. It is specialised in the hunting of wild boar and other large mammals. The breed was recognised by ENCI in 2003 and is fairly distributed in the central regions of Italy. The within- and between-breed genetic variation in many domestic animal species can be evaluated with microsatellites and other genetic markers. Microsatellite polymorphism analysis is commonly used to assess the genetic diversity and was the tool of choice in several studies on autochthonous breeds, such as Leroy *et al.* (2009) in 61 dog breeds found in France, Ciampolini *et al.* (2011) in the *Bracco Italiano* breed, Suárez *et al.* (2013) in the Canary island dog breeds and Mellanby *et al.* (2013) in dog breeds found in the England.

The aim of this study is to investigate the genetic differentiation between the two *Segugio* breeds through microsatellite markers. The two populations share the same geographic environment, and are found mainly in the Central and Northern parts of Italy. They are both hunting breeds, but the way of their utilisation in the sports is somewhat different; the SM is mainly used to trail and hunt down large game such as wild boars, while the SA is

more adapted to scent trail hares. The two breeds followed different selection patterns aimed at their ultimate envisaged use. The SM has to work as a member within packs of up to fifty dogs, and utilizing its developed sense of smell to scent and explore the territory to pick up trail on any wild boar in the vicinity. On the other hand the SA has to pick up the hare trail by sniffing the ground or the grass in pastures or cultivated fields. Since the two breeds perform in different manners, they are trained and selected on different parameters as a consequence, some of their morphological features are also different. While the SM breed is put together in a compact form, SA has a leaner composition and conformation; the coats are also quite different both in patterns and in colours (ENCI, 2015). This study will explore the possibility that the different selection strategies employed may have caused some appreciable detectable genetic differentiation between SA and SM even though both breeds are found in the same geographic regions and are utilized for similar purposes.

Materials and methods

In this study, 21 microsatellite markers were analysed in order to characterize the genetic

diversity of the within and between breeds. A total of 103 unrelated animals, 30 males and 24 females for SM (from 15 different owners) and 25 males and 24 females for SA (from 20 different owners), were sampled from animals that participated in hunting competitions and dog shows. Blood was collected from the jugular vein (5 mL from each animal, collected in vacutainer tubes containing EDTA as anticoagulant) and stored at -20°C until DNA extraction. Genomic DNA was extracted from whole blood using the Gen Elute Blood DNA kit (Sigma-Aldrich, St. Louis, MO, USA), and stored at -20°C until genotyping. The 21 microsatellites investigated belonged to a panel proposed by International Society of Animal Genetics (ISAG, 2005), for the parentage analysis. The microsatellites were genotyped by Genefast Srl (Valsamoggia, BO, Italy). The mean number of alleles per locus (MNA), observed heterozygosity (H_o) and expected heterozygosity (H_e) were estimated using the Excel microsatellite toolkit (Park, 2001). The number of private alleles in the different breeds was counted directly, an option provided for by the Convert software (Glaubitz, 2004). Test for deviations from Hardy-Weinberg equilibrium (dHWE) across all loci for each population were performed with GENEPOP 4.0.7, applying the exact test and using the Markov chain algorithm with default setting to calculate P-values (Guo and

Thompson, 1992). Weir and Cockerham's (1984) extension of Wright's F-statistics (F_{IS} , F_{IT} and F_{ST}) as well as the significances of the fixation indices were calculated with ARLEQUIN 3.11 software (Excoffier et al., 2010). Principal component analysis was performed with Nei's minimum distance (Nei, 1972) and estimates between the two groups were computed with GenAlEx software v. 6.5 (Peakall and Smouse, 2012). The assignment test and analysis of molecular variance (AMOVA) were also carried out with GenAlEx software v. 6.5 which offers both frequency-based and distance-based analyses. The algorithm implemented in the STRUCTURE software version 2.2 (Pritchard et al., 2000) was used to cluster individuals based on multilocus genotypes to assess population structure. The analysis involved an admixture model with correlated allele frequencies. One hundred independent runs were carried out with 300,000 iterations during the burn-in phase and 600,000 iterations for sampling from $2 \leq K \leq 5$ (K = number of clusters) to estimate the most likely number of clusters present in the dataset. The most probable number of population clusters was determined by calculating the distribution of K statistic as described by Evanno et al. (2005). The clustering pattern was visualised using the software DISTRUCT 1.1 (Rosenberg, 2004).

Table 1. Measures of genetic variability. Observed alleles number, fixation indices for each marker in the two breeds and polymorphic information content values.

Locus	N_A		F-statistics			PIC
	SM	SA	F_{IS}	F_{IT}	F_{ST}	
AHTK211	6	5	-0.100	-0.092	0.007	0.68
CXX279	7	7	0.031	0.034	0.003	0.76
AHT137	12	11	0.060	0.064	0.004	0.79
REN169O18	8	7	0.065	0.070	0.005	0.78
REN162C04	6	7	0.054	0.073	0.020*	0.68
REN54P11	8	8	0.114*	0.118*	0.005	0.75
FH2848	8	8	0.063	0.067	0.003	0.78
REN247M23	6	7	0.236*	0.242*	0.008	0.54
AHT121	10	12	0.044	0.059	0.016*	0.81
INRA21	7	7	0.010	0.020	0.010	0.78
AHTH130	13	11	0.014	0.022	0.008	0.84
INU030	6	7	-0.076	-0.072	0.004	0.71
FH2054	10	8	0.071*	0.083*	0.013	0.79
INU055	7	6	0.096*	0.109*	0.015	0.69
AHTH171	10	10	0.087*	0.097*	0.012	0.82
REN105L03	9	9	0.040	0.046	0.007	0.77
AHTK253	8	8	0.007	0.019	0.013	0.66
INU005	8	7	0.102*	0.129*	0.030*	0.61
REN64E19	8	9	-0.067	-0.058	0.009	0.67
REN169D01	8	7	-0.002	0.001	0.003	0.73
AHTH260	8	10	0.034	0.039	0.005	0.77
Mean	8.19±1.82		0.042±0.016*	0.051±0.016*	0.010±0.001*	0.73±0.07

N_A , number of observed alleles; PIC, polymorphic information content; SM, *Segugio Maremmano*; SA, *Segugio dell'Appennino*. *P<0.05.

Results and discussion

All of the analysed microsatellite markers were polymorphic (Table 1) and the average number of alleles per locus was 8.19. The markers with the lowest number of allelic variance were REN162C04, REN247M23, INU030 (6) for SM and INU055 (6) for SA; whereas the highest were AHTH130 (13) for SM and AHT121 for SA (12). The polymorphic information content (PIC) per locus ranged from 0.54 to 0.84, with an average of 0.73. According to Botstein *et al.* (1980), the PIC at all analysed loci was informative. These results are in agreement with the findings of Gagliardi *et al.* (2011) in Uruguayan dogs where among the nine markers analysed in the study, seven were highly polymorphic, with a PIC >0.7. Wright fixation indices per locus in the entire population are shown in Table 1; the mean F_{IS} value (inbreeding coefficient) was significantly different from zero (0.042) ($P < 0.05$) indicating the lack of heterozygosity in the studied sample. Similar values were reported by Ciampolini *et al.* (2011) ($F_{IS} = 0.061$) in the *Bracco Italiano* breed. The mean F_{IT} index (0.051; $P < 0.05$) shows that the gene flow within the population was altered at some stage, giving rise to a heterozygote deficiency in the total population. The mean F_{ST} fixation index, (0.010; $P < 0.05$), shows the absence of a significant genetic differentiation among the two breeds. The number of observed alleles for breed was 8.24 in SM and 8.14 in SA (Table 2). The values of observed and expected heterozygosity were similar in the two analysed breeds and higher than those estimated for other European dog breeds, where H_0 ranged from 0.55 to 0.67 whereas the H_E varied between 0.56 and 0.72 (Koskinen and Bredbacka, 2000). The number of private alleles is different in

the two populations (20 in SM and 18 in SA). All the loci were in H-W equilibrium except for two in SM (REN247M23, REN105L03) and three in SA (REN54P11, REN247M23, AHTH171). The F_{IS} values (inbreeding coefficient) in SM and SA breeds were both significantly different from zero (0.05746 and 0.04312, respectively) indicating a certain level of heterozygote deficiency most likely due to non-random mating or population subdivision. It must be noted that, although artificial insemination is not used in dog breeding, the selective pressure is still significant due to the fact that only a few sires are mated extensively, either for morphological reasons or because of their hunting performances. Furthermore, it has to be taken into consideration that the high number of pups born per litter increases the number of relatives in the population and

therefore encourages the probability to increase inbreeding. For each of the three models summarised in Table 3, AMOVA analysis does not highlight significant differences in terms of genetic variation (F_{ST} values); this result confirms the already reported values of heterozygosity and of the number of alleles detected in the samples. Moreover, AMOVA analysis shows that the larger portion of the variation is found within individuals (93%) rather than among the breeds (1%). Principal coordinate analysis (PCA) was also performed and the first 2 components accounted for 38.42% of the observed variation (Figure 1). The PCA analysis indicated that there was no substantial variability among the two breeds as shown by the almost complete overlap between them. This is also confirmed by the low value (0.063) of genetic distance between SM and

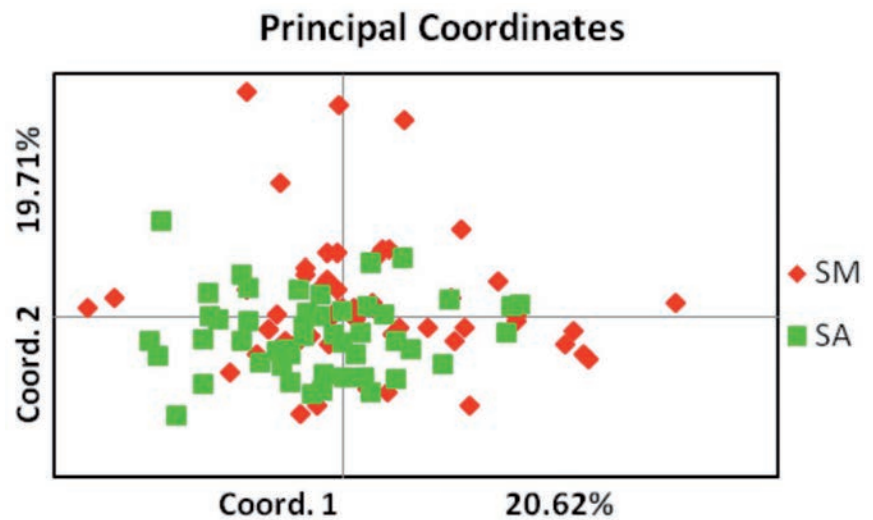


Figure 1. Principal coordinate analysis obtained by Nei's minimum distance. SM, *Segugio Maremmano*; SA, *Segugio dell'Appennino*.

Table 2. Mean number of observed alleles, mean observed and expected heterozygosity, private alleles, number of markers deviated from the Hardy-Weinberg equilibrium per breed and inbreeding coefficient per breed.

Breed	Acronym	Sample size	N_A	H_0	H_E	Private alleles	HWE	F_{IS}
<i>Segugio Maremmano</i>	SM	54	8.24±1.89	0.73±0.01 ^a	0.78±0.01 ^a	20	2	0.05746*
<i>Segugio dell'Appennino</i>	SA	49	8.14±1.80	0.73±0.01 ^a	0.77±0.02 ^a	18	3	0.04312*

N_A , number of observed alleles; H_0 , observed heterozygosity; H_E , expected heterozygosity; HWE, Weinberg equilibrium per breed. ^aSuperscript letters in H_E and H_0 columns indicate no significant differences (Tukey's HSD test, $P < 0.05$). *Significantly different at $P < 0.05$.

Table 3. Results of hierarchical AMOVA for the two studied breeds *Segugio Maremmano* and *Segugio dell'Appennino*.

Source of variation	df	Sum of squares	Expected means square	%	F_{ST}	P
Among populations	1	16.36	16.36	1	0.016	0.001
Among individuals	101	873.79	8.65	6		
Within individuals	103	787.50	7.64	93		

SM, *Segugio Maremmano*; SA, *Segugio dell'Appennino*.

SA. The population assignment diagram in Figure 2 shows again an almost complete overlap between the two studied breeds; this situation could be due to the fact that both breeds have a common ethnological source from the Italian *Segugio*. Only a few SM individuals show a peculiar positioning when compared to the rest of the population (Figures 1 and 2). It could be assumed that these individuals are genetically quite different from the rest of the

population, probably due to uncontrolled cross-breeding practices. STRUCTURE analysis confirmed the general features observed in the PCA and in the population assignment analysis. The results indicate that, for the two populations analysed, the most likely grouping of the pool of dog populations is at $K=3$. The STRUCTURE analysis highlighted a third population substructure probably due to a genetic introgression from different *Segugio* breeds.

Conclusions

The results obtained by microsatellite analysis show that the genetic variability of the *Segugio* populations investigated in this work are comparable to other dog populations. It is possible to speculate that some factors influenced the normal gene flow among the animals, giving rise to an overall heterozygote deficiency in the total population.

Our results highlight the non-significant differences among the two breeds in terms of genetic differentiation. The presence of population substructure is probably due to a genetic introgression from different *Segugio* breeds.

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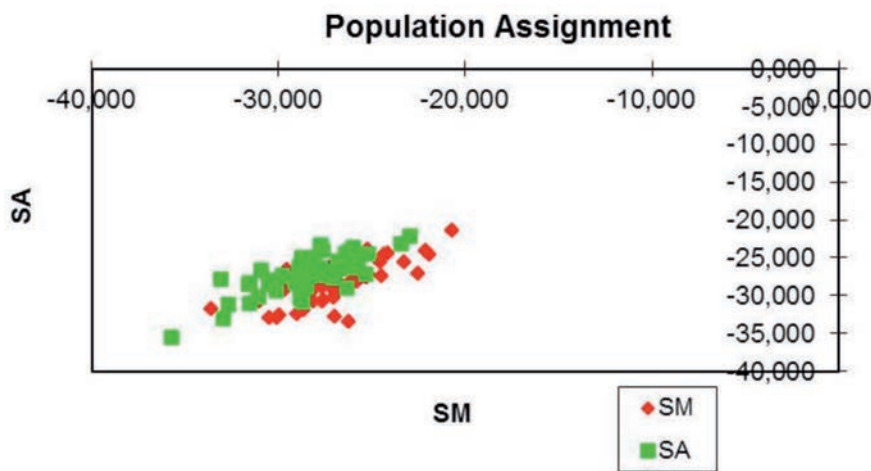


Figure 2. Results of population assignment. SM, *Segugio Maremmano*; SA, *Segugio dell'Appennino*.

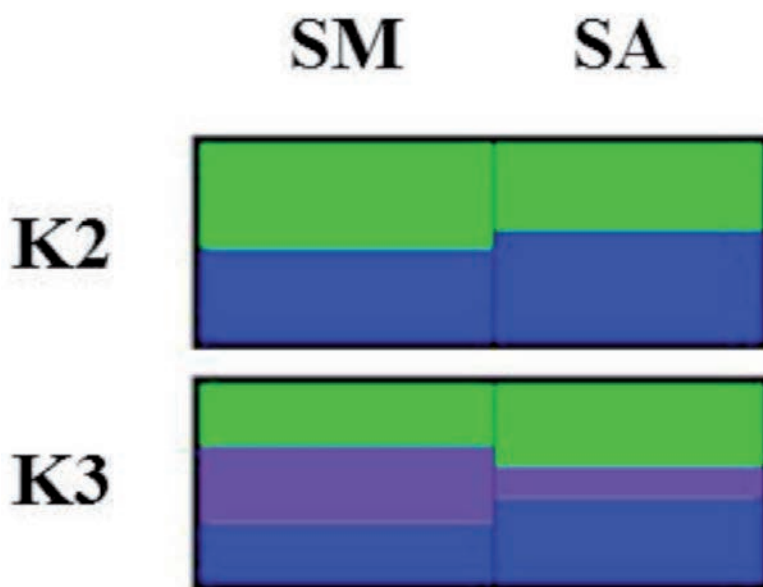


Figure 3. Structure cluster analysis of the two dog populations. SM, *Segugio Maremmano*; SA, *Segugio dell'Appennino*.

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