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Original article

Differentiation of the Italian wolf and the domestic dog based on microsatellite analysis

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Abstract – The Italian wolf is in the process of regaining the Alpine region which comes into conflict with the extensive sheep keeping practiced in Switzerland during the summer As in Switzerland, the wolf is a protected species, the government reimburses losses caused by wolves Therefore we wanted to know whether the Italian wolf could be distinguished from the domestic dog by microsatellite analysis if DNA samples of the predators could be secured The evaluation of combined genotypes for the microsatellites CanBern6, CPH4, CPH7, CPH9, CPH12, CPH22 and ZuBeCa1 made it possible to identify an individual as either a domestic dog or an Italian wolf The assignment of an individual to either one of the two populations is based on the logarithm of the likelihood ratio of an individual being an Italian wolf rather than a domestic dog, given a specific combined genotype The distribution of the Italian wolf combined genotypes ($n = 42$) is clearly distinct from the distribution of the domestic dog combined genotypes ($n = 90$) The likelihood ratio for the "worst" Italian wolf combined genotype was 2.3 E+5 and for the "worst" domestic dog combined genotype was 3.8 E − 5.

Italian wolf / domestic dog / microsatellite / genotype / likelihood ratio

Résumé – Différenciation entre le loup italien et le chien domestique par l'analyse de microsatellites. Le loup italien est en train de s'installer dans les Alpes et entre en conflit avec la transhumance estivale des moutons pratiquée en Suisse. Étant donné que le loup est protégé en Suisse, le gouvernement rembourse les pertes causées par le loup Nous voulions savoir s'il est possible de distinguer le loup italien du chien domestique par l'analyse de microsatellites, en se basant sur des échantillons d'ADN appartenant au prédateur En tenant compte de génotypes

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combinés des microsatellites CanBern6, CPH4, CPH9, CPH12, CPH22 et ZuBeCa1, il est possible d'identifier un sujet comme chien domestique ou loup italien L'attribution d'un sujet à l'une des deux populations se base sur le logarithme du rapport de vraisemblance d'un sujet étant plutôt un loup italien qu'un chien domestique avec un génotype combiné spécifique donné. La distribution des génotypes combinés du loup italien ($n = 42$) est nettement distincte de la distribution des génotypes combinés du chien domestique ($n = 90$) Le rapport de vraisemblance pour le loup italien avec le génotype combiné le plus défavorable s'élève à $2.3\,E+5$, et, pour le chien domestique avec le génotype combiné le plus défavorable, il est de $3\,8\,E-5$

loup italien / chien domestique / microsatellite / génotype / rapport de vraisemblance

1. INTRODUCTION

The wolf was eradicated in Switzerland towards the end of the 19th century [1]. Thereafter the very few confirmed reports on wolf sightings were believed to involve animals escaped from enclosures. In recent years, however, evidence has accumulated indicating that wolves of Italian origin are venturing into Switzerland on a regular basis. One or two individuals were sighted between the years 1995 and 1996, one was shot and a car killed one in 1998 and another one was sighted in 1999. The two dead animals were identified as Italian wolves based on mitochondrial DNA analysis (Taberlet, personal communication). The expansion of the Italian wolf into the Swiss Alps interferes with the extensive sheep keeping during the pasture season. Each summer more than 200 000 sheep, which is about half of the total, roam, more or less unattended, the Alpine pastures. During this period sheep are lost to predators and with the reappearance of the wolf these numbers have noticeably increased. As in Switzerland wolves may not be hunted, the government has to reimburse these losses. Therefore the agencies involved want to have a means to clearly identify the culprits, since besides wolves, domestic dogs can also cause similar damage.

The goal of this study was to evaluate whether microsatellite markers would allow us to distinguish between Italian wolf and domestic dog genotypes.

2. MATERIALS AND METHODS

2.1. Animals

The two populations used for the estimation of genotype frequencies consisted of 70 Italian wolves and 90 domestic dogs. We assumed that the Italian wolves were not closely related since they originated from different places throughout Italy. Each of the 90 dogs represented a different breed. In addition, representatives of *Bernese Mountain Dog* ($n = 9$), *Boxer* ($n = 8$), *German Shepherd* ($n = 7$), *Belgian Shepherd* ($n = 7$), *Landseer* ($n = 6$) and *Newfoundland* ($n = 18$) were genotyped. Three wolves of different origins were also included: one from a farm in Poland, one from a game park in Switzerland

and one of unknown origin. In the case of the Italian wolf, muscle tissue samples in ethanol were used for DNA extraction, in all other cases EDTA-blood was used.

2.2. Genotyping

Of the 38 microsatellites evaluated (data not shown) the following seven were selected for the analysis: CanBern6 [2], CPH4, CPH7, CPH9, CPH12 [6], CPH22 (upper primer: 5′TCTTTCATTTACATTTTTGGCTCA3′; lower primer: 5′GCCCCAAAATCCGTGTGT3′, Fredholm, personal communication) and ZuBeCa1 [9] With the exception of CPH22, which has not yet been mapped, these loci are not linked according to the radiation hybrid map by Priat *et al.* [7] and unpublished data of the DogMap consortium. PCR amplifications were carried out in 12 μL containing 2 μL DNA solution (Chelex 100, BioRad, Hercules, CA, USA or High Pure PCR Template Preparation Kit, Boehringer Mannheim, Mannheim, Germany), 2.5 pmol of each primer, 0.25 mM of each dNTP, 1 × PCR buffer with 1 5 mM $MgCl_2$ (Appligene, Gaithersburg, MD, USA) and 0.35 units Taq polymerase (Appligene) in a Perkin 9600 or 9700 thermocycler. PCR was performed using the following touch-down program [3]: initial denaturation for 5 min at 94 °C, two cycles each of 30 s at 94 °C, 30 s in the touch-down range of 68 °C to 60 °C for ZuBeCa1, 59 °C to 50 °C for CPH4 and 63 °C to 55 °C for all the others, from the highest down to the lowest annealing temperature and 30 s at 72 °C, followed by 14 cycles where only the lowest annealing temperature was used and the final extension at 72 °C for 30 min. Allele sizes were determined on 8% denaturing polyacrylamide gels using a LI-COR DNA sequencer model 4200 (LI-COR, Lincoln, NE, USA)

2.3. Data analysis

If in population A_1 the genotype frequencies F_{1i} at n unlinked loci are known, the probability of the combined genotype G for an individual can be calculated:

$$P\langle G|A_1\rangle = \prod_{i=1}^{n} F_{1i}.$$

In the same way, the combined genotype probabilities for the same n loci can be calculated for populations A_2, A_3 to A_r. The overall combined genotype probability in all k populations would then be:

$$P\langle G\rangle = \sum_{r=1}^{k} P\langle A_r\rangle * P\langle G|A_r\rangle$$

where the A's are pairwise mutually exclusive populations. We did not allow for hybridization between different populations. According to Bayes' theorem

the posterior probability of an individual with the combined genotype G to belong to a specific population A_j is:

$$P\langle A_j|G\rangle = \frac{P\langle A_j\rangle * P\langle G|A_j\rangle}{\sum_{r=1}^{k} P\langle A_r\rangle * P\langle G|A_r\rangle}.$$

We will restrict our considerations to the two populations of interest, that is Italian wolf (W) and domestic dog (D), which makes this posterior probability:

$$P\langle \mathrm{W}|G\rangle = \frac{P\langle \mathrm{W}\rangle * P\langle G|\mathrm{W}\rangle}{P\langle \mathrm{W}\rangle * P\langle G|\mathrm{W}\rangle + P\langle \mathrm{D}\rangle * P\langle G|\mathrm{D}\rangle}.$$

Since we do not know the prior probabilities of an individual to belong to either one of the two populations we assume them to be equal, $P(\mathrm{W}) = P(\mathrm{D})$. The ratio R of the respective posterior probabilities then turns out to be a likelihood ratio [4], which allows us to express our confidence in assigning an individual to one and not to the other population based on its combined genotype·

$$\frac{P\langle \mathrm{W}|G\rangle}{P\langle \mathrm{D}|G\rangle} = \frac{P\langle G|\mathrm{W}\rangle}{P\langle G|\mathrm{D}\rangle} = \frac{L\langle \mathrm{W}|G\rangle}{L\langle \mathrm{D}|G\rangle} = R.$$

The allele frequences for each locus were estimated based on all genotypes actually observed. In order to avoid allele frequencies in a population being zero, one homozygous genotype of the respective length was added to the double of the observed number of genotypes. The tests of Hardy-Weinberg equilibrium and linkage disequilibrium were performed with the computer program GENEPOP [8].

3. RESULTS

The allele frequencies of the seven loci differed considerably between the Italian wolf and the domestic dog for the seven loci analyzed (Fig. 1). The combined genotypes were assigned to classes of $\log R$ that increase in steps of 2 (Fig. 2). The italian wolf and domestic dog were separated into two distinct distributions, where R for the "worst" Italian wolf combined genotype was $2.3\,\mathrm{E}+5$ ($\log R$ 4 to 6) and where R for the "worst" domestic dog combined genotype was $3.8\,\mathrm{E}-5$ ($\log R$ -6 to -4). All domestic dog controls' combined genotypes were found in the domestic dog combined genotype distribution and the three control wolves in the Italian wolf distribution.

Linkage disequilibrium and Hardy-Weinberg equilibrium were calculated based on the genotypes actually observed. All loci in the Italian wolf population were in Hardy-Weinberg equilibrium with the exception of CPH7 ($P = 0\ 0188$). However, the Italian wolf sample shows a global concordance with Hardy-Weinberg equilibrium ($P = 0.61$) across all seven loci [5]. For the domestic dog population Hardy-Weinberg equilibrium was not tested because this population did not meet the prerequisite for the Hardy-Weinberg equilibrium. Of the 21 linkage disequilibrium tests in the Italian wolf population, only CanBern6-CPH7 ($P = 0.00438$) and CPH4-ZuBeCa1 ($P = 0.00184$) were significant.

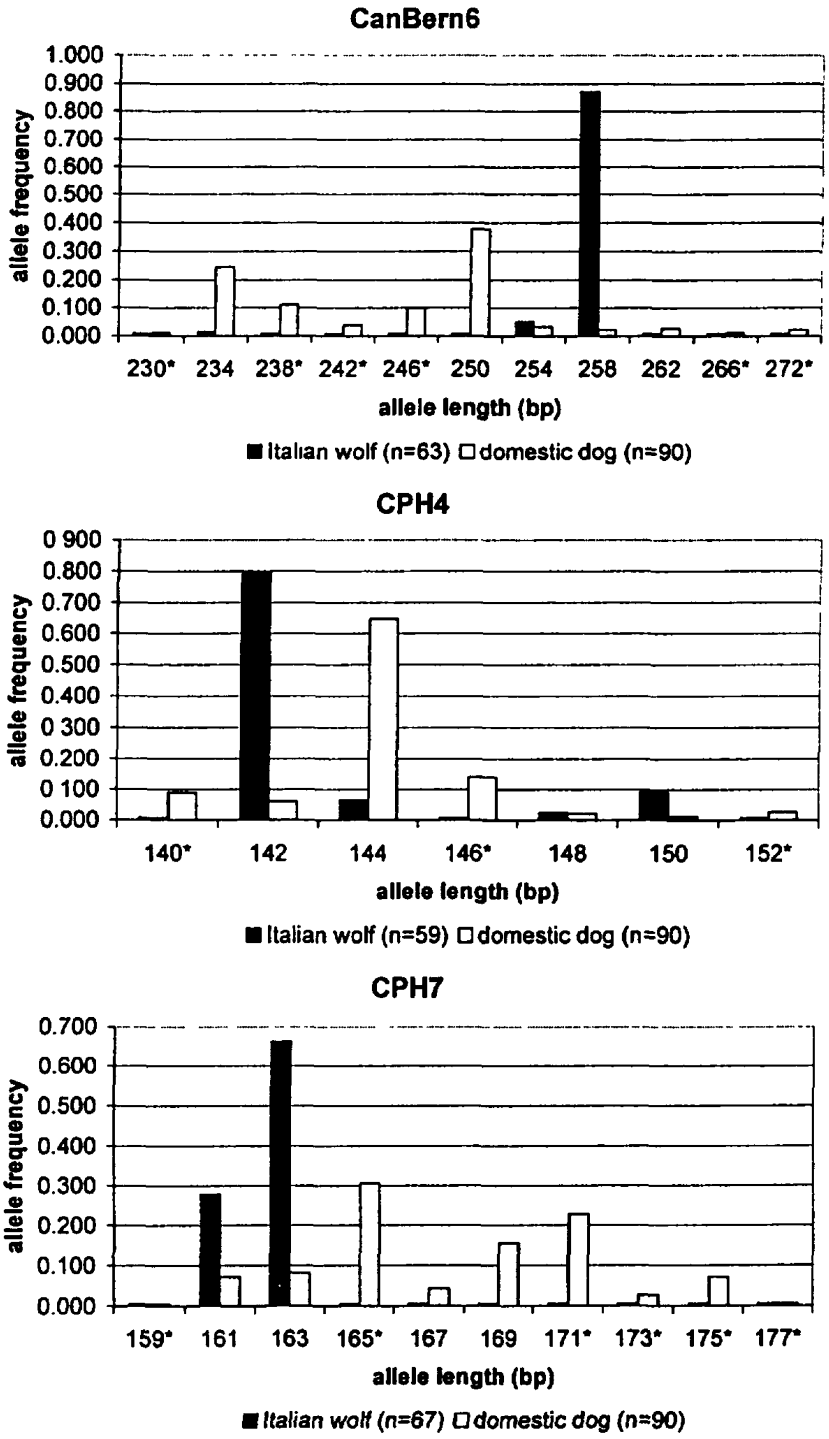

Figure 1. Comparison of allele frequencies of the Italian wolf and domestic dog. In the case where an allele length was not observed one homozygous genotype was added for the Italian wolf (*) or domestic dog (°).

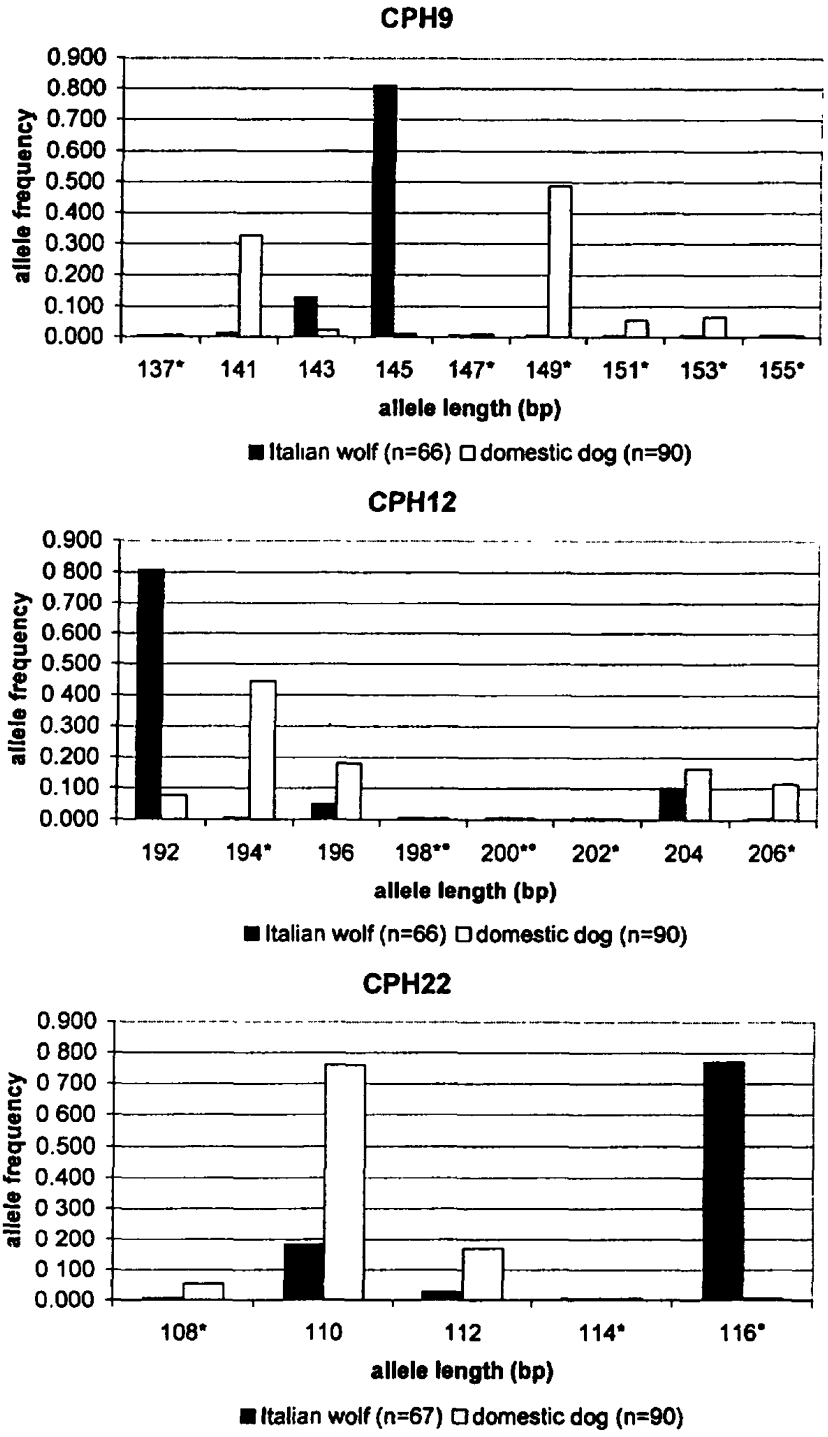

Figure 1. Continued.

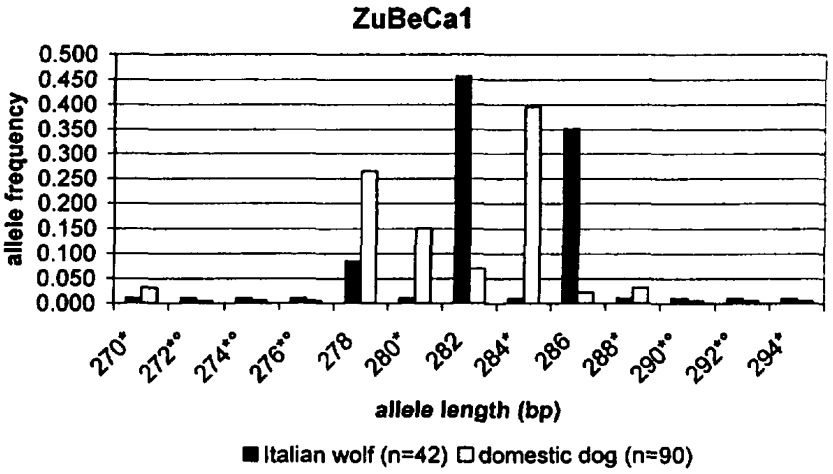

Figure 1. Continued.

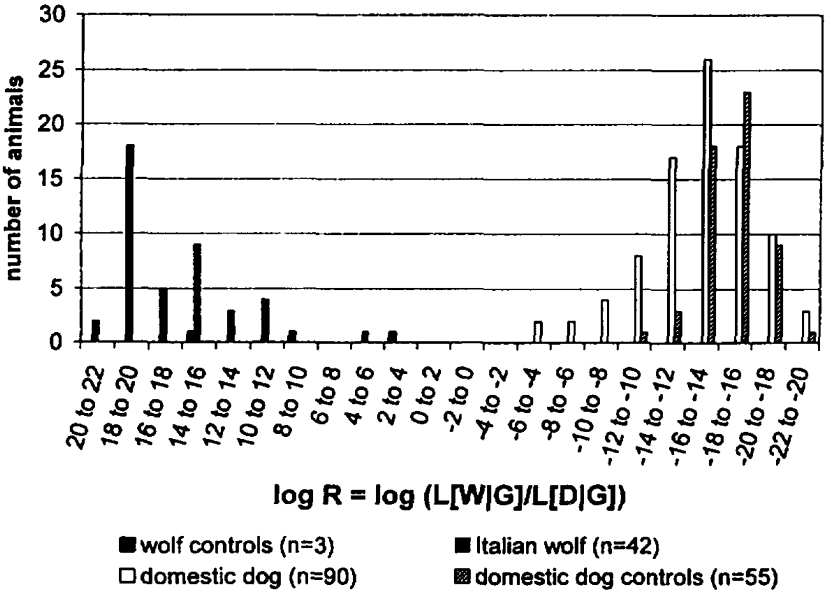

Figure 2. Distribution of the combined genotypes of 7 loci in log(L[W|G]/L[D|G]) classes.

4. DISCUSSION

The Italian wolf samples originated from animals that were killed on the road, poisoned or poached throughout Italy. The linkage disequilibrium at two pairs of loci that were not linked, and the Hardy-Weinberg disequilibrium at one locus could be attributed to an insufficient sample size, biased sampling or the occurrence of hybridization with the domestic dog. But since we had no prior knowledge of relationships among the animals or hybridization occurrences,

we assumed these samples to represent the Italian wolf population. Of the 70 Italian wolf samples only 42 allowed us to amplify all seven loci. The main reason for not producing 70 complete combined genotypes was the restricted amount of DNA at our disposal. Our domestic dog samples represented a synthetic population since they included one individual each from 90 different breeds. We chose this population because there was no evidence that specific breeds could be made responsible for killing sheep in the part of the Alps where Italian wolves are returning. Although the domestic dog population is far from ideal, it may adequately represent the situation in the Alps. The area concerned is in the process of being re-occupied by Italian wolves, inhabited by dogs of various breeds and mongrels, and frequently visited by tourists bringing along their dogs, representing the whole range of breeds. The combined genotypes of our domestic dog and Italian wolf samples were sufficiently distinct to separate them into two distinct populations (Fig. 2). The addition of missing alleles by adding virtual homozyotes prevents $P(G/\mathrm{W})$ and $P(G/\mathrm{D})$ from becoming zero, thus making the prediction robust. This measure allowed us to take into account the limited sample sizes and the rationale was that the next genotyped animal would be one carrying an allele not observed in the one population but present in the other population. A test of our heuristic approach with 55 dogs representing 6 different breeds clearly identifies them as domestic dogs. The three control wolves were identified as Italian wolves which indicates that the wolf as such is more distinct from the domestic dog than among their own different populations. Our investigation demonstrates that the Italian wolf can be distinguished from the domestic dog based on microsatellite analysis if DNA samples are available. The practical application would require the accounting for individuals that are neither Italian wolves nor domestic dogs. In Switzerland, this would require the accounting for captive wolves by establishing their DNA profiles for the loci involved. In addition, the monitoring of individuals of breeds with recent introgression of the wolf, such as the Czekoslovakian Wolfdog and Saarlos Wolfdog, may be indicated.

ACKNOWLEDGEMENTS

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