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Allozyme variability in the Italian wolf (*Canis lupus*) population

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Multilocus protein electrophoresis was used to estimate genetic variability in a sample of 38 Italian wolves (*Canis lupus*). Percentage of polymorphic loci was $p = 10.0$ per cent (four polymorphic loci out of 40 examined), and average observed heterozygosity was $\overline{Ho} = 0.028$. Genotypes were in Hardy–Weinberg equilibrium. Electrophoretic analysis does not indicate a significant reduction of genetic variability at nuclear gene loci following at least one century of isolation from other European populations and demographic fluctuations suggested by recent range contraction and expansion. These findings are compared with published allozyme and mitochondrial DNA data for dogs, Canadian wolves, and introgressed wolf × coyote populations from Minnesota and Isle Royale (U.S.A.).

Keywords: allozyme electrophoresis, *Canis lupus*, conservation genetics, dog, genetic variability, wolf.

Introduction

Systematic persecution of wolves in western Europe during the last two centuries has reduced their previously continuous distribution to patchy remnants. The Iberian and Italian populations have survived prolonged isolation, unlike other populations in Europe (Council of Europe, 1990). Cagnolaro and co-workers (1974) date the confinement of wolves south of the Po River to the turn of the last century.

Zimen & Boitani (1975) suggested that the Italian wolves declined until the end of the 1960s, when they were estimated to survive in 10 isolated populations scattered along the Apennines. The authors, extrapolating counts from three such 'islands' to the remainder, concluded that about 100 wolves survived in Italy in 1973. Two years later the species was listed as fully protected nationwide. According to Boitani (1984), diminished persecution eventually allowed wolves to disperse into larger ranges and increase population size. Interbreeding with free-ranging dogs, abundant in the central and southern Apennines, was indicated as both a threat to the integrity of the wolf gene-pool, and as an aid to the recovery of the population, when effective in counteracting inbreeding depression and genetic drift (Boitani, 1984). A track count conducted in winter 1983 in the same three 'wolf-islands' surveyed in 1973 and extrapolated to the known range, based on specimens found dead, suggested a total of 220 wolves south of latitude 43° 20′ (Fig. 1), partitioned into two populations along latitude 41° (Boitani, 1984).

In contrast, Cagnolaro *et al.* (1974) document a lower degree of population decline and fragmentation. According to their findings, territorial and dispersing wolves in 1972 ranged almost uninterrupted along the Apennines between latitude 44° and 39° (Fig. 1). That population was large enough to support an average of 63 legal-kills per year during the decade 1960–70, a figure contrasting with the low population size estimated by Zimen & Boitani (1975). Francisci & Guberti (1993) analysed specimens found dead and suggested that the Italian wolf population remained quite stable. Its slow recovery over the last 20 years added approximately 1° latitude to both the northern and southern extremes of the range assessed in 1972 by Cagnolaro *et al.* (1974). The present population is estimated at 300 (Boitani *et al.*, 1989), or 400 (Boscagli, 1991).

The genetic structure of the Italian wolf population, although relevant for the conservation of the species (Boitani, 1992), has not been investigated previously. In this paper we study genetic variability at structural genes in a sample of wolves from central-northern Italy, using multilocus protein electrophoresis. We dis-

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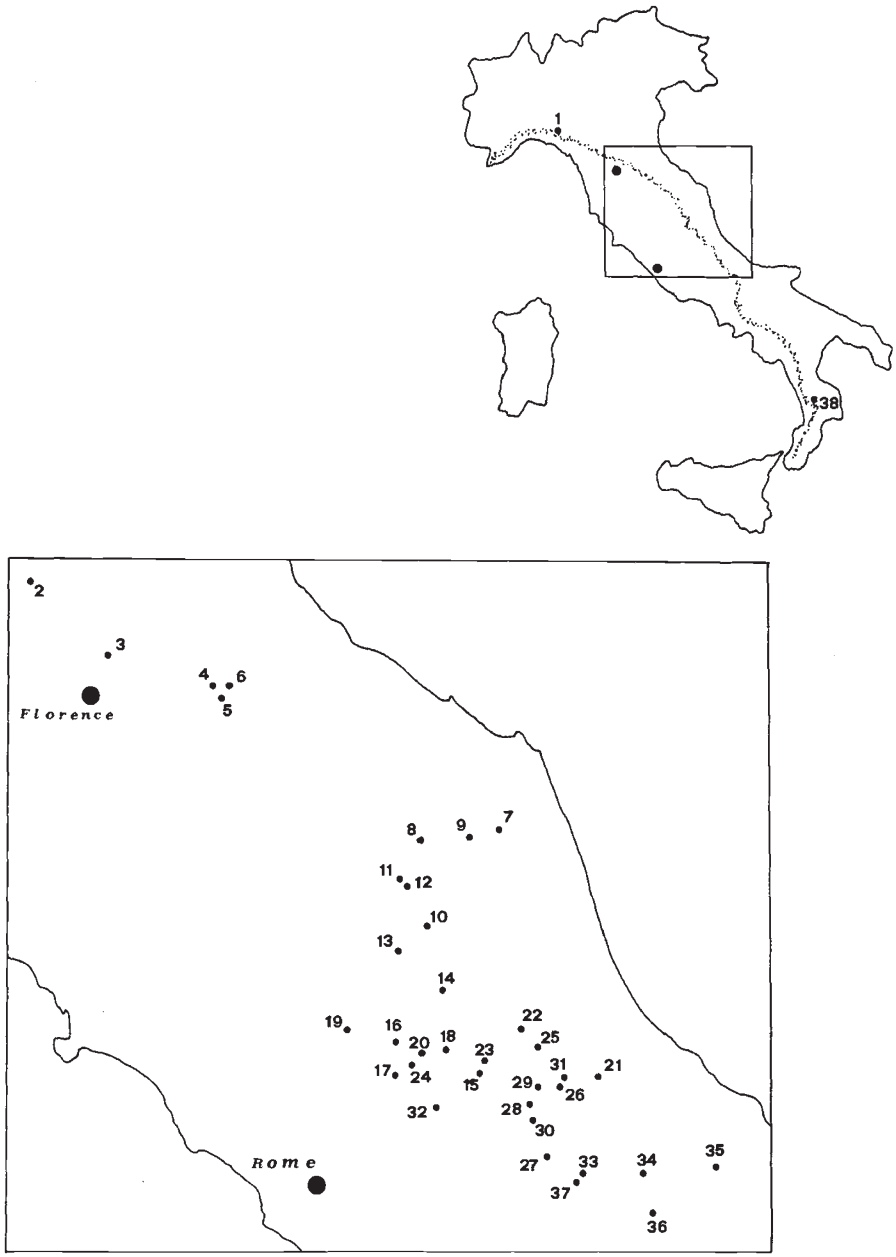

Fig. 1 Distribution of the wolves sampled. Individual genotypes are listed in Table 2. The inset shows the area from which most of the samples were collected. Dusted line represents divide of Apennine range.

cuss our results in the context of available demographic information for wolves in Italy.

The possible effects of over one century of isolation from other European populations, and those possible over the last decades with either an undivided, rather persistent population, or one that fragmented to near-annihilation and rapidly recovered, are also taken into account. Estimating rates of erosion of genetic variability and increase in homozygosity, as well as detecting introgression of dog genes into the wolf population, add information valuable for effective wolf conservation in Italy.

Materials and methods

Tissue samples (liver and heart) were obtained from 38 wolves, most of them collected in central-northern peninsular Italy (Fig. 1) from 1986 to 1991. These specimens were illegally shot (45 per cent), snared (15 per cent), or poisoned (25 per cent), while 15 per cent were killed by vehicles. Samples were stored at −80°C until processing. About 1 g of each tissue was separately homogenized in 1 ml of 0.01 M Tris/HCl, *p*H 7.5, 0.001 M Na_2 EDTA, 0.001 M β-mercaptoethanol buffer, and centrifuged for 15 min at 13,000 r.p.m., at

4°C. Clear supernatants were diluted in 1 volume of a 40 per cent glycerol solution, aliquoted in microtiter plates, and frozen at −80°C until use. Vertical (VPAGE) and horizontal (HPAGE) polyacrylamide gel electrophoresis (7.5 per cent monomers concentration in the resolving gels) were used to study allozyme variation at 40 loci (Table 1). Several enzymes were resolved in more than one buffer system. Staining recipes were adapted from Harris & Hopkinson (1976).

Electromorphs were presumed to have a simple genetic basis, and were considered as alleles. Alleles were coded according to their mobility from the origin, with the most anodal allele designated as 'a'. Loci were coded with numbers, −1 being the most anodal, usually corresponding to the cytoplasmic isozymes, and −2 corresponding to the mitochondrial isozymes.

The program BIOSYS-1 (Swofford & Selander, 1989) was used to compute allele frequencies, effective allele number (A), percentage of polymorphic loci (P; 1 per cent criterion), and heterozygosity (H) values. Agreement with Hardy-Weinberg expectations was tested by Chi-square (Sokal & Rohlf, 1981) and exact probability (Weir, 1990) tests.

Results

Four loci (*GPI, DIA-1, GOT-1, MPI*) out of 40 resolved, were polymorphic in the Italian wolf samples. Individual genotypes and allele frequencies are shown in Table 2 and Table 3, respectively. Heterozygote patterns corresponded to expectations based on the known quaternary structure of these enzymes in vertebrate species (Harris & Hopkinson, 1976). *GPI* showed three alleles, while the other loci showed two. The average effective allele number was $\overline{A}=1.1$ (over all loci), and 2.3 (polymorphic loci only). Percentage of polymorphism was P= 10.0 per cent; average observed and expected heterozygosities were $\overline{Ho}=0.028$ and $\overline{He}=0.029$, respectively (Table 4). The greatest con-

Table 1 List of the studied protein loci and electrophoretic conditions

Buffer system	Reference	Locus (E.C.N.)
Discontinuous Tris/glycine (*p*H 8.3 VPAGE)	Davis, 1964	*ACP* (3.1.3.2) *AMY* (3.2.1.1) *MDH-1* (1.1.1.37) *ME-1* (1.1.1.40) *FUM* (4.2.1.2) *GLUD* (1.4.1.3) *GDA* (3.5.4.3) Albumin (*ALB*) Proteins (*HPT-1*, -2, -3) *SOD-1*, -2 (1.15.1.1) *CK* (2.7.3.2) *LDH-1*, -2 (1.1.1.27)
Discontinuous Tris/glycine (*p*H 8.5 VPAGE)	Jolley & Allen, 1965	Haemoglobin (*HB-1*, -2) *PGM* (2.7.5.1) *GPI* (5.3.1.9) *GOT-1* (2.6.1.1)
Tris/barbital (*p*H 7.0 VPAGE)	Williams & Reisfeld, 1964	*α GPDH* (1.1.1.8) *ME-2* (1.1.1.40) *IDH-1*, -2 (1.1.1.42) *EST-1*, -2, -3, -4, -5 (3.1.1.1) *LA-PEP-A* (3.4.11) *LGG-PEP-B* (3.4.11)
Phosphate (*p*H 7.0 HPAGE)	Harris & Hopkinson, 1976	*6PGD* (1.1.1.44)
Tris/borate/EDTA (*p*H 8.6 HPAGE)	Harris & Hopkinson, 1976	*NADH DIA-1* (1.6.2.2) *NADPH DIA-2* (1.6)
Tris/phosphate (*p*H 8.3 HPAGE)	Harris & Hopkinson, 1976	*MPI* (5.3.1.8)
LiOH/boric acid (*p*H 7.2 HPAGE)	Harris & Hopkinson, 1976	*NP* (2.4.2.1)
Tris/citrate (*p*H 6.8 VPAGE)	Harris & Hopkinson, 1976	*AK* (2.7.4.3) *ALD* (4.1.2.13)
Phosphate/citrate (*p*H 5.9 VPAGE)	Harris & Hopkinson, 1976	*MDH-2* (1.1.1.37)

Table 2 Individual genotypes at polymorphic loci for 38 Italian wolves (locations shown in Fig. 1)

Wolf number	Enzyme locus			
	GPI	*GOT-1*	*MPI*	*DIA-1*
1	bb	aa	aa	bb
2	bc	ab	ab	ab
3	bb	aa	aa	aa
4	bb	aa	aa	bb
5	bb	aa	ab	bb
6	bb	aa	ab	ab
7	bb	aa	aa	ab
8	bb	aa	aa	bb
9	bc	aa	aa	bb
10	bb	aa	aa	aa
11	bb	aa	ab	bb
12	bb	aa	aa	bb
13	bb	ab	aa	bb
14	bb	aa	ab	bb
15	bb	aa	aa	bb
16	bb	aa	aa	aa
17	bc	aa	aa	ab
18	bb	aa	bb	ab
19	bb	aa	ab	bb
20	bb	aa	ab	aa
21	bb	ab	ab	ab
22	bc	aa	aa	bb
23	bb	aa	ab	ab
24	bb	aa	aa	bb
25	bb	aa	ab	aa
26	bb	aa	aa	ab
27	bb	aa	ab	aa
28	ab	aa	ab	bb
29	bb	aa	bb	ab
30	bc	aa	ab	bb
31	bc	aa	aa	bb
32	bb	aa	ab	bb
33	bb	aa	ab	bb
34	bb	aa	ab	bb
35	bc	aa	ab	ab
36	bb	ab	ab	bb
37	bb	aa	ab	ab
38	bb	aa	bb	ab

Table 3 Allele frequencies at polymorphic loci in a sample of 38 wolves from central-northern Italy

Loci		*GPI*	*GOT-1*	*MPI*	*DIA-1*
Alleles	a	0.013	0.947	0.671	0.316
	b	0.895	0.053	0.329	0.684
	c	0.092	0.000	0.000	0.000

tributions to average heterozygosity were from *MPI* and *DIA-1* (average heterozygosity $\overline{Ho} = 0.45$). Commonest alleles for *GPI* and *GOT-1* were at a frequency higher than 0.89, and their heterozygosities were lower than 0.20. All polymorphic loci were in Hardy–Weinberg equilibrium, and heterozygote genotypes were scattered over all the sampled area (Fig. 1 and Table 2).

Allozyme variability in the Italian wolves was compared (Table 4) with recently published data on wolf populations from Canada (Kennedy *et al.*, 1991), Minnesota and Isle Royale, U.S.A., (Wayne *et al.*, 1991). Minnesota wolves belong to a wolf × coyote (*Canis latrans*) hybrid population, as determined by mtDNA analysis (Lehman *et al.*, 1991). All the Isle Royale wolves possess the same mtDNA haplotype, which is of coyote origin (Lehman *et al.*, 1991). A low percentage of polymorphic loci (P = 8.0; 1 per cent criterion) was observed in the small population (only 14 survivors in 1990) on Isle Royale. This island population showed less than 40 per cent polymorphic loci compared with the nearby mainland Minnesota hybrid population (P = 20.0 per cent). The observed low genetic diversity of the Isle Royale wolves can result (at least in part) from the very low sample ($n = 7$). Wolves sampled in Canada showed P = 17.9 per cent (1 per cent criterion), slightly lower than the Minnesota hybrid population. The Italian wolves were intermediate between the Isle Royale and the mainland North America populations (Table 4). Heterozygosity values were similar in the Italian, Canada, and Isle Royale populations ($\overline{Ho}$ ranging from 0.028 to 0.040), and were 40–50 per cent ($P < 0.001$, *t*-test) lower than in the Minnesota hybrid population (Table 4).

Discussion

Generalized hybridization with free-ranging dogs was postulated (Boitani, 1984) as one of the major factors allowing the supposedly quick recovery of wolves in Italy during the late 1970s–early 1980s, and is still regarded as the main threat to the species' survival at present (Boitani, 1992).

The few studies comparing allozyme variability among canids could not detect any single-locus fixed difference between dog and wolf (Simonsen, 1976; Fisher *et al.*, 1976; Ferrel *et al.*, 1980; Braend & Roed, 1987). Genetic distances generated through multilocus protein electrophoresis were low, indicating close phylogenetic relationships among coyote, jackal (*Canis aureus*), wolf, and dog (Seal, 1975; Wayne & O'Brien, 1987). Evolutionary divergence among these canids was accordingly estimated to be fairly recent (2 million years; Wayne & O'Brien, 1987).

Table 4 Percent polymorphic loci (P%, 0.01 criterion), observed heterozygosity (Ho), and expected heterozygosity (He) in Italian and North American wolf populations

	Wolf		Introgressed wolf × coyote (a)	
Population:	Italy (b) ($n = 32$)	Canada (c) (188)	Isle Royale (7)	Minnesota (33)
p% (0.01)	10.0	17.9	8.0	20.0
Ho	0.028	0.030	0.040	0.061
He	0.029	NC (d)	0.039	0.087

(a) Wayne *et al.*, 1991. (b) Sampling area shown in Fig. 1. (c) Kennedy *et al.*, 1991. (d) NC = not computed.

Loci that we have found polymorphic in Italian wolves, were also polymorphic in other wolf populations (Kennedy *et al.*, 1991; Wayne *et al.*, 1991), and in several dog breeds (Meera Khan *et al.*, 1973; Weiden *et al.*, 1974; Fisher *et al.*, 1976; Arnold & Bouw, 1985). Genetic variability of gene–enzyme systems has been studied in large samples of many and differing dog breeds. According to these studies we may infer average values of P ranging from 10 to 20 per cent, and $\overline{Ho}$ ranging from 0.030 to 0.040 in domestic dogs, roughly compare with estimates found in wolf populations (Kennedy *et al.*, 1991; Wayne *et al.*, 1991; this study). Allozyme variability may not detect hybridization of wolves with dogs, unless exhaustive samples of sympatric dogs and wolves are available from areas of possible hybridization. Careful estimation of allele frequencies at many polymorphic loci, or use of species-specific DNA markers is needed to investigate possible gene flow between dog and wolf.

Preliminary restriction fragment length polymorphism (RFLP) analysis showed that mtDNA was monomorphic in 14 Italian wolves collected by the Italian Institute for Wildlife Biology (INFS) during 1986–1989 (Wayne *et al.*, 1992). The single mtDNA Italian wolf haplotype was distinct from other European and North American wolf and dog samples (Wayne *et al.*, 1992). Recent RFLP analysis of an expanded sample ($n = 30$) confirmed the monomorphism of mtDNA of Italian wolves, and discovered the existence of fixed sequence differences with free-ranging dogs (Randi *et al.*, in preparation). Therefore, mtDNA findings offer no evidence of widespread maternal introgression of dog genes into the present Italian wolf gene pool. If wolf × dog hybridization is sex biased, mtDNA cannot be used as a marker. Dog × wolf hybridization is known for wolves of both sexes, although published reports of hybridization in nature refer to she-wolves (Boitani, 1984; Zimen, 1978, for one case in Italy; Mendelssohn, 1982, for one in Israel). Examples of hybrid populations or evidence for introgression, however, are not known. Interbreeding was not apparent during a 4-year study of sympatric wolves and dogs in Abruzzo. Feral dogs adjacent to, or included in, wolves' territories, survived by minimizing contact with the latter species (Francisci *et al.*, 1992).

Though Italian and Canadian wolves show similar values of heterozygosity and percentage of polymorphic loci (Table 4), the former are monomorphic at *ME-1* and *PGM-1*, where the latter are polymorphic, with variants at low frequencies (Kennedy *et al.*, 1991). It may be expected that a relatively large and uniformly distributed sample of wolves in Italy would increase the probability of detecting polymorphic loci, although this might be counteracted by both isolation and demographic fluctuations.

Available information support our findings that wolves in northern-central peninsular Italy behave as a single panmictic population in Hardy–Weinberg equilibrium. Heterozygote genotypes were randomly distributed throughout the sampled area (Table 2), while allozyme variability does not indicate inbreeding or population subdivision (Wahlund effect; Hartl & Clark, 1989). Because we were not able to obtain specimens from the southern Apennines we cannot comment on the degree of local genetic divergence. The habitat continuum which the Apennines still offers to wolves, particularly to dispersers, suggests the species in Italy has probably remained panmictic through its range (*cf.* Ciucci & Boitani, 1991). Finer population subdivisions will have to be assessed by sampling different packs and by analysis of fast-evolving DNA sequences (mtDNA and DNA fingerprinting).

Our findings indicate that the bulk of genetic variability in wolves in northern-central Italy has not been lost. Moderate bottlenecks may cause loss of rare and low-frequency alleles or mtDNA haplotypes (Nei *et al.*, 1975). If mtDNA monomorphism is confirmed on a

larger sample size, more complex metapopulation dynamics should be taken into account. For example, isolation from continental populations effective since before the last 100 years (Cagnolaro *et al.*, 1974), might have significantly reduced the species' variability in Italy, even in the absence of bottlenecks, when combined with a steady population decrease (Wayne *et al.*, 1991). Repeated local extinction and recolonization (Boitani, 1992) might have greatly reduced the effective population size and sped up the erosion of genetic variability due to random drift (Gilpin, 1991) in the Italian wolf population. Availability of larger samples of Italian and other European populations, may allow a better understanding of the population genetics of wolves in Italy and the extent of geographical differentiation in Europe.

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