Genetic evidence for multiple events of hybridization between wolves and domestic dogs in the Iberian Peninsula

RAQUEL GODINHO,* LUIS LLANEZA,*† JUAN C. BLANCO,‡ SUSANA LOPES,*
FRANCISCO ÁLVARES,* EMILIO J. GARCÍA,† VICENTE PALACIOS,†
YOLANDA CORTÉS,‡ JAVIER TALEGÓN§ and NUNO FERRAND*¶
CIBIO—Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal, †A.RE.NA Asesores en Recursos Naturales, S.L., c/Perpetuo Socorro, 12—Entres. 2-B, 27003 Lugo, Spain, ‡Proyecto Lobo CBC, C/Manuela Malasaña 24, 28004 Madrid, Spain, §C/Judería 33, 29800 Toro (Zamora), Spain, ¶Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n. 4169-007 Porto, Portugal

Abstract

Hybridization between wild species and their domestic counterparts may represent a major threat to natural populations. However, high genetic similarity between the hybridizing taxa makes the detection of hybrids a difficult task and may hinder attempts to assess the impact of hybridization in conservation biology. In this work, we used a combination of 42 autosomal microsatellites together with Y-chromosome microsatellite-defined haplotypes and mtDNA sequences to investigate the occurrence and dynamics of wolf–dog hybridization in the Iberian Peninsula. To do this, we applied a variety of Bayesian analyses and a parallel set of simulation studies to evaluate (i) the differences between Iberian wolves and dogs, (ii) the frequency and geographical distribution of hybridization and (iii) the directionality of hybridization. First, we show that Iberian wolves and dogs form two well-differentiated genetic entities, suggesting that introgressive hybridization is not a widespread phenomenon shaping both gene pools. Second, we found evidence for the existence of hybridization that is apparently restricted to more peripheral and recently expanded wolf populations. Third, we describe compelling evidence suggesting that the dynamics of hybridization in wolf populations is mediated by crosses between male dogs and female wolves. More importantly, the observation of a population showing the occurrence of a continuum of hybrid classes forming mixed packs may indicate that we have underestimated hybridization. If future studies confirm this pattern, then an intriguing avenue of research is to investigate how introgression from free-ranging domestic dogs is enabling wolf populations to adapt to the highly humanized habitats of southern Europe while still maintaining their genetic differentiation.

Introduction

The natural history of European populations of grey wolf (*Canis lupus*) has been characterized by a dramatic decline in numbers during the past few hundred years

Correspondence: Raquel Godinho, Fax: +351 252 661780;
E-mail: rgodinho@mail.icav.up.pt

(Boitani 2003). By the end of the 19th century, the species apparently survived only in the southern peninsulas (Iberia, Italy and the Balkans) and in Eastern regions, where it persisted till legal protection status was established in most European countries in late 20th century, leading to the recent and well-documented wolf population expansion (Boitani 2003). Decline in numbers, historical population fragmentation and

disruption of gene flow are well-known triggers of genetic impoverishment and inbreeding in natural populations and certainly increase the risk of extinction in wolves and many other species (Allendorf & Luikart 2007). An example of this situation has been documented for Italian wolves, in which values of genetic diversity (as measured by heterozygosity) are clearly below those exhibited in dense and continuous populations from Russia, Alaska or Canada (Roy *et al.* 1994; Vilà *et al.* 2003; Verardi *et al.* 2006). In addition, recent wolf expansion to humanized areas has led to several other threats. In particular, hybridization between wolves and free-ranging dogs recently became a growing concern for conservationists in Europe and a subject addressed in many research programmes (Vilà & Wayne 1999; Boitani 2003).

Hybridization between wild species and their domestic counterparts may represent a major threat to natural populations. In fact, the spread of 'domestication genes' into natural populations may disrupt local adaptation and/or increase genetic homogenization, eventually leading to the extinction of species through introgressive hybridization (Rhymer & Simberloff 1996). Additionally, the imbalance between population numbers of domesticated and wild forms—the former typically exceeding the latter by several orders of magnitude—may facilitate unidirectional gene flow and suggests that hybridization is a serious threat to the genetic integrity of natural populations. Randi & Lucchini (2002) indicate that in Italy the number of free-ranging dogs exceeds the number of wolves by a factor of 10^3 or more. It is thus possible that this imbalance, together with the low-density and fragmented nature of wolf populations, increases the risk of hybridization with dogs. Unfortunately, the high genetic similarity of dogs and wolves makes detecting hybrids difficult, which may explain why evidence reporting the occurrence of hybrid wolves in Europe has been scarce (Andersone *et al.* 2002; Randi & Lucchini 2002; Vilà *et al.* 2003), and the significance of this issue for wolf conservation remains largely unknown.

In the last few years, new data reported for wolf populations in both Scandinavia and Italy have contributed to a better understanding of the frequency of hybridization and its directionality. Vilà *et al.* (2003) combined the use of mtDNA, Y-chromosome and autosomal molecular markers and identified a single hybrid resulting from a cross between a Scandinavian female wolf and a male dog. In a second example, Randi and colleagues used between 18 and 20 autosomal microsatellites to suggest that the percentage of hybrid wolves in Italy could be as high as 5% (Randi & Lucchini 2002; Verardi *et al.* 2006). While their strategy of using sets of linked microsatellites proved successful and apparently indicated a higher than expected percentage of hybridization in Italian wolves, it

was unfortunate that the lack of both maternally and paternally inherited markers prevented a detailed analysis of the dynamics of hybridization. In addition, Vähä & Primmer (2006) used simulations to show that the efficient detection of hybrids requires many more microsatellite loci than are currently employed in most conservation genetic programmes. For example, even with a moderate average F_{st} value of 0.12, at least 24 loci are required for the efficient detection of F1 hybrids. However, if the aim is also to separate backcrosses from purebred parental individuals, then the genotyping effort must be much larger, involving at least 48 loci. These results suggest that the magnitude of hybridization may be systematically underestimated, clearly indicating the need for further research with additional loci.

Presently, the Iberian Peninsula holds more than 2000 wolves, essentially concentrated in a large and continuous population in the northwestern region, but also in two isolates, one in Andalusia, Southern Spain, and other south of the river Douro, in Central Portugal (Blanco & Cortés 2002; Álvares *et al.* 2005). In Iberia, wolves and dogs have coexisted for a long time in a profoundly modified landscape that humans and livestock have been shaping for several thousand years (López-Merino *et al.* 2009, 2010). Previous studies have shown that Iberian wolves use agricultural habitats, frequently occurring close to human settlements and feeding mostly on livestock (Cuesta *et al.* 1991; Llaneza *et al.* 1996; Vos 2000; Blanco & Cortés 2007). It is thus likely that the peculiar biology of the wolf in the highly humanized and disturbed Iberian habitats favours contact with feral and free-ranging dogs, possibly resulting in extensive hybridization (Petrucci-Fonseca 1982; Blanco *et al.* 1992). However, except Sundqvist (2008), no comprehensive study has investigated the occurrence and dynamics of wolf–dog hybridization in the Iberian Peninsula. In this work, we used a total of 42 autosomal microsatellites, six Y-linked microsatellites, and mitochondrial DNA haplotypes to address the following questions. (i) What are the levels of genetic diversity of Iberian wolves and how do they compare with other European populations? (ii) How clear is the clustering and differentiation of Iberian wolves and dogs? (iii) How frequent and geographically distributed is hybridization between wolves and dogs? (iv) What classes of hybrids (e.g. F1s and backcrosses) can be identified and what are the implications for wolf conservation in Iberia?

Material and methods

Sample collection and laboratory procedures

A total of 408 biological samples (blood, tissue or buccal swabs) were obtained from 208 putative Iberian wolves,

196 dogs and four potential hybrid animals (the latter was based on morphological and behavioural evidence). Our wolf sample represents most of the continuous distribution area in Iberia and the isolated population in Central Portugal (Fig. 1) and was obtained from dead animals (mainly road kills and hunting) collected between 1996 and 2009 ($n = 188$), from wild animals captured for scientific purposes ($n = 19$) and from one captive animal thought to have been captured in the wild. The dog samples comprise 54 feral dogs collected across the wolf range and 152 purebred dogs representing four Iberian Molossoid cattle dog breeds (Serra da Estrela, $n = 36$; Castro Laboreiro, $n = 28$; Cão de Gado Transmontano, $n = 29$; and Rafeiro do Alentejo, $n = 32$) and German Shepherd ($n = 17$). Additionally, four samples of potential hybrids classified by their previous observation in apparently mixed dog/wolf packs and/or their unusual morphological traits, especially a distinctive coat colour, were included in the analysis (samples L81, L82, L318 and L405; see Fig. 1 and Table 2 for geographical locations and Fig. 2 for morphological traits for some of these animals). Total genomic DNA was extracted using QIAGEN DNeasy Blood & Tissue Kit or QIAamp DNA Micro Kit depending on the quality and quantity of sample available.

Individual multilocus genotypes were determined using a set of 42 dog autosomal microsatellites (see Table S1, Supporting information for description of loci), which all proved to be polymorphic in the Iberian wolf. Eighteen loci were amplified using a commercial kit (FINNZYMES) following manufacturer's instructions, and the remaining 24 loci were amplified in three multiplex reactions (MP1, MP2 and MP3; see Table S1, Supporting information for allocation of loci to each multiplex) using the Multiplex PCR Kit (QIAGEN)
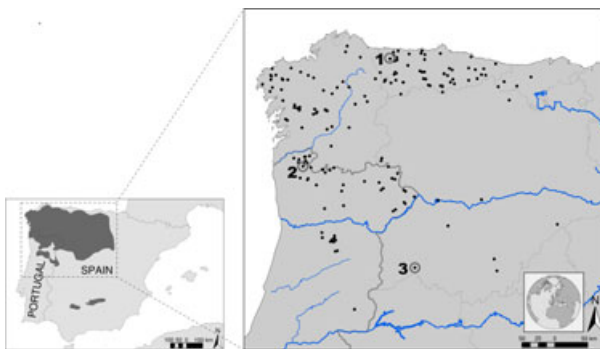
following polymerase chain reaction (PCR) conditions given in the manufacturer's instructions and with the annealing temperature set to 56 °C (MP1 and MP2). Thermocycling for MP3 used a touchdown profile with the annealing temperature decreasing from 60 °C to 57 °C in eight cycles, followed by 30 cycles with constant annealing temperature set to 57 °C. Additionally, six Y-linked microsatellites (MS34A, MS34B and MS41B from Sundqvist *et al.* 2001; and 650–79.2, 650–79.3 and 990–35 from Bannasch *et al.* 2005) were genotyped for male samples in a single multiplex using Multiplex PCR Kit (QIAGEN) with an annealing temperature of 60 °C. All amplifications were performed in a 10-µL volume in Bio-Rad thermal cyclers (MyCycler and iCycler) always using negative controls to monitor possible contaminants. PCR products were separated by size on an ABI3100xl genetic analyser using the 350ROX size standard. Alleles were determined using GENEMAPPER 4.0 (Applied Biosystems) and checked manually. In order to thoroughly verify and confirm the observed genotypes, 20% of wolf and dog samples were reamplified and reanalysed for each locus, resulting in complete concordance among replicates.

Fig. 1 Location of sampling sites for wolves in Iberian Peninsula. The three sites where morphologically identified hybrids were collected are identified with circles: (1) Western Asturias, Spain; (2) Minho, Portugal; (3) Castilla y Léon, Spain. Inset: Iberian Peninsula and wolf distribution area in dark grey (from Álvares *et al.* 2005).

Fig. 2 Three animals killed in the same pack in Sierra de Penouta, Western Asturias, Spain. On top is a 'pure' wolf; in the middle, the hybrid L81; and at the bottom, the hybrid L82 (individual assignment to wolf population of 100%, 43% and 31%, respectively).

To address the direction of hybridization, hybrid individuals were scored for their mitochondrial lineage using universal primers Thr-L 15926 and DL-H 16340 to amplify a 425-bp fragment of the mitochondrial control region, as described by Vilà *et al.* (1999). Successful amplifications were purified using enzymes exonuclease I and Shrimp alkaline phosphatase and sequenced with BigDye chemistry (Applied Biosystems). Electropherograms were verified and aligned using SEQSCAPE 2.5 (Applied Biosystems).

Data analysis

Autosomal microsatellite diversity was evaluated separately for dogs and wolves (hybrid individuals were excluded) based on allele frequencies, mean number of alleles per locus (Na), number of private alleles and observed (Ho) and expected (He) heterozygosities for each locus using ARLEQUIN 3.5 (Excoffier & Lischer 2010). The same software was used to evaluate deviations from Hardy–Weinberg equilibrium and to test pairwise linkage disequilibrium for all loci (16 000 permutations) based on the exact test of Guo & Thompson (1992). Significance levels were adjusted using the sequential method of Bonferroni for multiple comparisons in the same data set (Rice 1989). Population differentiation was assessed by Fisher's exact test, analogues of pairwise mean F_{st} (Weir & Cockerham 1984) and analysis of molecular variance (AMOVA, Michalakis & Excoffier 1996), using ARLEQUIN 3.5.

A first exploratory analysis to visualize patterns of genetic differentiation between Iberian wolves and dogs was performed in GENETIX 4.05 (Belkhir *et al.* 2004) using a factorial correspondence analysis.

Bayesian clustering analysis implemented in the program STRUCTURE 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003) was used to assign individuals to two populations ($K = 2$) and to identify hybrids between wolf and dog. As each individual may have ancestry in more than one parental population, analyses were performed using the admixture model with correlated allele frequencies. No priors of individual identification were used. STRUCTURE was run with five repetitions of 10^6 MCMC iterations following a burn-in period of 10^5 steps in order to guarantee the achievement of similar posterior probabilities of the data in each run and to ascertain confidence in the model fit. We assessed the average proportion of membership (Q_i) of wolf and dog populations to the inferred clusters, and the individual membership proportion q_i of each sample to those two clusters. Additionally, NEWHYBRIDS 1.1 (Anderson & Thompson 2002) was used to achieve a more detailed analysis of admixture proportions and hybrids ancestry, by inferring the posterior probability assignment (q) of each individual

to six genotype frequency classes: wolf, dog, F1, F2, backcross with wolf and backcross with dog.

The inherent drawback of the Bayesian approach is that the validity of the assumed priors and the efficiency of analysed loci cannot be statistically assessed; consequently, simulations have to be implemented for each empirical data set in order to evaluate the statistical limit of that particular study (Nielsen *et al.* 2006). Following this, we assessed the power of the markers and models used in the admixture analyses to distinguish among parental and hybrid classes and established the range of q-values expected for all possible admixed generations by simulating both parental and hybrid genotypes in HYBRIDLAB 1.0 (Nielsen *et al.* 2006). Based on individual multilocus genotypes, the program initially estimates, locus by locus, allele frequencies for each of the parental wild and domestic populations. Afterwards, multilocus F1 hybrid genotypes are created by randomly selecting one allele from each of the two populations, according to their frequency distribution (Nielsen *et al.* 2006). Simulations of other hybrid classes (F2 and backcrosses genotypes) can be computed by the successive use of simulated genotypes as starting-point populations. We selected 100 parental wolves and 100 parental dogs to generate 50 genotypes of each parental and hybrid class: wolf, dog, F1, F2 and respective first-generation backcrosses with wolf and dog. With $K = 2$, simulated genotypes were then used in STRUCTURE without any prior non-genetic information, with the goal of assessing the efficiency of the admixture analyses in estimating the proportion of hybrids in the simulated data set (see Barilani *et al.* (2007) for further details). Similarly, simulated genotypes were used in NEWHYBRIDS to assess the efficiency of the analysis in allocating simulated individuals to their a priori known class (parents or one of the four hybrid classes).

Results

Genetic diversity at 42 nuclear microsatellites

A total of 42 autosomal microsatellites was analysed for 208 putative wolves, 196 dogs and four potential hybrids based on morphological traits from Iberian Peninsula (Fig. 1). All loci were polymorphic, showing between 4 and 26 alleles per locus and values of expected heterozygosity ranging from 0.498 (FHC2079) to 0.898 (AHT121; see Tables S1 and S2, Supporting information for details on each locus). Genetic diversity showed marked differences between Iberian wolves and dogs. Iberian wolves exhibit the lowest values for all genetic diversity measures analysed: for example, the mean number of alleles per locus excluding low-frequency alleles (frequency ≤ 0.05) was $Na_{wolf} = 3.9$

($\pm$0.238) and Na$_{dog}$ = 5.3 ($\pm$0.185), while the mean expected heterozygosity was He$_{wolf}$ = 0.617($\pm$0.031) and He$_{dog}$ = 0.755 ($\pm$0.013) (both measures were significantly different as determined by a paired *t*-test, $P < 0.001$) (Table 1). While ascertainment bias could contribute to explain these differences (see for example Vowles & Amos (2006) and Ellegren *et al.* (1997) for a discussion on this subject in humans/chimpanzees and cattle/sheep, respectively), we believe that the multiple events of domestication and introgression during dog history are a more likely explanation for their higher genetic diversity values when compared to those exhibited by the isolated Iberian wolf population.

Some of the markers used in this study map to the same dog chromosome, but the large physical distance occurring between them should be enough to prevent linkage. In fact, when testing for linkage disequilibrium (LD) for all pairs of loci, we found 188 pairwise combinations in wolf and 113 in dogs that were significant (significance probability level $P < 0.05$, Bonferroni corrected for 1722 comparisons) but only 75 of these comparisons were common to both wolf and dog. When verifying the physical location for these 75 pairwise combinations, none of them were at the same chromosome. The explanation for these results is certainly related to demographic factors that can inflate LD, including inbreeding and population structure, both because dog breeds are artificial populations that violate the assumptions of random breeding and because Iberian wolves are known to have a certain degree of population structure (R. Godinho *et al.*, unpublished data).

Population genetic differentiation

Although most of the variation was found within individuals (73.4%) and a less important fraction among individuals within populations (10.0%), results reflect

Table 1 Variability at autosomal microsatellite loci for Iberian wolf and dog. Hybrid individuals were excluded from this analysis

	Iberian wolf	Dog
Sampling size	204	196
Mean no. of alleles per locus	6.405 ($\pm$0.434)	8.762 ($\pm$0.560)
Mean no. of alleles per locus ($P \geq 0.05$)	3.905 ($\pm$0.238)	5.310 ($\pm$0.185)
No. of private alleles	51	150
No. of private alleles ($P \geq 0.05$)	29	92
Mean expected heterozygosity	0.617 ($\pm$0.031)	0.755 ($\pm$0.013)

distinct genetic entities between Iberian wolves and dogs. Over all loci, a noteworthy proportion of the total genetic variation was partitioned between wolves and domestic dogs ($F_{st} = 0.166$; $P < 0.001$). Loci that contribute more for the observed differentiation of Iberian wolf and dog were DBar1, AHTk211, C14.866 and AHT103, which exhibited F_{st} values of 0.538, 0.431, 0.333 and 0.323, respectively. By contrast, loci that revealed the lowest levels of differentiation among wild and domestic populations were FH2161, VWF, AHT121 and FH2001, which presented F_{st} values of 0.022, 0.032, 0.042 and 0.051, respectively (see Table S2, Supporting information for F_{st} values on other loci).

Results of an exploratory analysis with factorial correspondence analysis (FCA) followed the same trend: FCA scores of all individuals were graphically presented in a dimensional plot defined by two principal axes that explain, cumulatively, 8.7% of the total genetic variability (Fig. 3). The plotting shows an evident separation between Iberian wolves and dogs, revealing a clear genetic differentiation between them. Outliers of the two clusters are found in between wolf and dog populations and correspond to the four samples initially identified as possible hybrids based on morphological traits (L318, L405, L81, L82), in addition to another sample (L64) also detected as a hybrid by the Bayesian analysis (see next section).

Admixture patterns

Bayesian admixture analyses performed with STRUCTURE without any prior on the origin of individuals and allowing two clusters clearly suggested the presence of two differentiated biological groups that sharply splits dogs from Iberian wolves (Fig. 4A). All dogs were probabilistically assigned to cluster I, with an average proportion of membership of $Q_I = 0.995$, while Iberian wolves were mostly assigned to cluster II with $Q_{II} = 0.990$ (Table 3, Fig. 4A). However, there were eight individuals in the Iberian wolf cluster, including the four samples previously morphologically identified as possible hybrids (Fig. 4A, Table 2), which were partially assigned to both clusters with individual $q_i < 0.85$ (Table 3). Assuming that probabilistic assignments below that threshold indicate admixture, a minimum of 4% of the Iberian wolves in this study showed signals of introgressive hybridization.

Bayesian analysis of simulated genotypes, including parental classes, F1, F2 and the respective first-generation backcrosses, revealed that parental individuals were assigned to their correct class with an average q_i of 0.982 for Iberian wolf and 0.975 for dog, and 90% credibility intervals (CI) were higher than 0.85 in both groups of 50 simulated parental individuals (Fig. 4B).
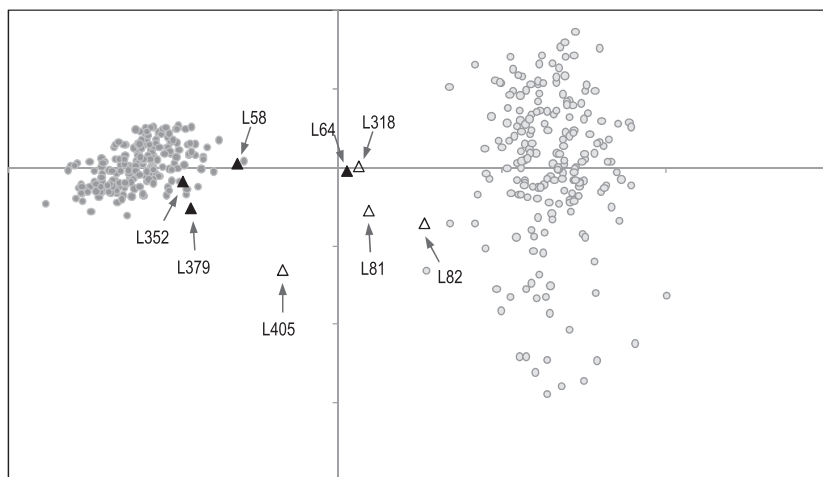
Fig. 3 Plot of individual scores of the first two synthetic variables of a factorial correspondence analysis (first factor on X axis and second factor on Y axis) using the set of 42 microsatellites. Light circles indicate wolf samples, dark circles indicate dog samples, open triangles indicate hybrids identified by morphology and dark triangles indicate hybrids based on Bayesian analysis.
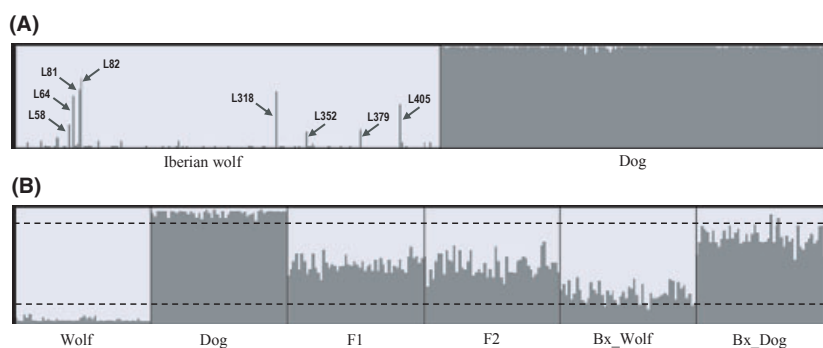

Fig. 4 Probabilistic assignment to the genetic clusters inferred by the Bayesian analysis with $K = 2$ of (A) Iberian wolves and dogs and (B) simulated genotypes belonging to six distinct classes, wolf, dog, F1, F2, backcross with wolf (Bx_Wolf) and backcross with dog (Bx_Dog) simulated using HYBRIDLAB v. 1.0. Each individual is represented by a vertical line fragmented into two sections that are relative to their membership proportion in the genetic clusters: Iberian wolf (light grey); dog (dark grey). The horizontal dashed lines represent the threshold probability of 85% defined using the simulated genotypes in NEWHYBRIDS used to assign each individual to a single population.

Table 2 Description of samples that exhibited admixture between wolf and domestic dog, and their information on mitochondrial and Y-chromosome genomic compartments

Sample	Sex	Date	Locality, Region	Country	mtDNA	Y
L58	Male	2002	Boal, West Asturias	Spain	Wolf	Wolf
L64	Female	1999	Acebo, West Asturias	Spain	Wolf	–
L81	Male	2004	Penouta, West Asturias	Spain	Wolf	Dog
L82	Female	2004	Penouta, West Asturias	Spain	Wolf	–
L318	Male	2008	South of Salamanca, Castilla y Léon	Spain	Wolf	Dog
L352	Male	2007	La Bobia, West Asturias	Spain	Wolf	Dog
L379	Male	2008	Mondoñedo-Lugo, Galicia	Spain	Wolf	Dog
L405	Female	2009	Mezio-Arcos de Valdevez, Minho	Portugal	Wolf	–

Average membership coefficient of simulated F1 individuals was $q_{iF1} = 0.513$ (0.247 < CI < 0.753), and for simulated F2, the average membership coefficient achieved was $q_{iF2} = 0.529$, while credibility intervals were slightly wider than for F1 (0.212 < CI < 0.788). Backcross-simulated genotypes showed an average

Table 3 Individual assignment (q_i) and inferred ancestry of wolves and dogs, and of the eight individuals identified with admixed origin. q_i values were calculated in STRUCTURE considering two clusters and without prior population information. Ancestry was calculated in NEWHYBRIDS using six genotype classes (wolf, dog, F1, F2, BxWolf (backcross with wolf) and BxDog (backcross with dog)) and prior population information for all individuals but the eight with admixed origin. STRUCTURE 90% probability intervals are shown in brackets. Values in bold indicate the most likely hybrid class

	Individual assignment		Inferred ancestry					
	Wolf	Dog	Wolf	Dog	F1	F2	BxWolf	BxDog
Wolf	0.995 (0.976–1.000)	0.005 (0.000–0.024)	0.999	0.000	0.000	0.000	0.001	0.000
Dog	0.004 (0.000–0.023)	0.996 (0.977–1.000)	0.000	0.999	0.000	0.000	0.000	0.001
L58	0.770 (0.635, 0.886)	0.230 (0.114, 0.365)	0.000	0.000	0.000	0.008	**0.992**	0.000
L64	0.486 (0.332, 0.637)	0.514 (0.363, 0.668)	0.000	0.000	**0.956**	0.035	0.005	0.004
L81	0.427 (0.250, 0.605)	0.573 (0.395, 0.750)	0.000	0.000	0.095	**0.460**	0.002	**0.443**
L82	0.305 (0.168, 0.452)	0.695 (0.548, 0.832)	0.000	0.000	0.171	0.108	0.000	**0.721**
L318	0.451 (0.292, 0.611)	0.549 (0.389, 0.708)	0.000	0.000	**0.969**	0.022	0.002	0.007
L352	0.848 (0.723, 0.950)	0.152 (0.050, 0.277)	0.016	0.000	0.000	0.001	**0.983**	0.000
L379	0.819 (0.699, 0.920)	0.181 (0.080, 0.301)	0.000	0.000	0.000	0.000	**1.000**	0.000
L405	0.573 (0.430, 0.709)	0.427 (0.291, 0.570)	0.000	0.000	**0.902**	0.011	0.087	0.000

membership coefficient of $q_{iBxWolf} = 0.774$ and $q_{iBxDog} = 0.769$, and the credibility intervals were respectively $0.447 < CI < 0.968$ and $0.424 < CI < 0.992$. These results suggest that our set of microsatellites may detect 100% of F1 and F2 individuals with a threshold of 0.85. However, the power of this set of loci to detect a first-generation backcross with wolf decreases to 84%, and to 76% in the case of a backcross with dog. These values indicate that 16% and 24% of simulated backcross hybrids, for wolf and dog, respectively, were above the threshold of 0.85 (Fig. 4B).

The evaluation of the statistical limits of our results with the Bayesian analysis suggests that we can confidently accept that the eight samples that were partially assigned to both clusters with individual $q_i < 0.85$, and that were exceptions to the sharp differentiation observed between the two clusters, are, in fact, hybrids. These samples originated in seven wild individuals, five of them from Asturias, Spain (L58, L64, L81, L82 and L352), one from Castilla y Léon, Spain (L318), and one from Minho, Portugal (L405); the eighth sample came from a captive individual in Galicia, Spain (L379), supposedly caught from a wild pack (Table 2, Fig. 2).

Assignment to hybrid classes

To further explore our results, we investigated the probability of each of these eight hybrid individuals belonging to a strict hybrid class (F1, F2 or backcrosses) using the framework of Bayesian model-based clustering (Anderson & Thompson 2002). The results of this method indicated that six out of the eight individuals exhibit posterior probabilities above 90% for a single hybrid class (Table 3). Samples L64, L318 and L405

were assigned to the F1 class with probabilities of 0.956, 0.969 and 0.902, respectively, while samples L58, L352 and L379 were assigned to the backcross with wolf class with probabilities of 0.992, 0.983 and 1.000, respectively. The two remnant individuals (L81 and L82), while confirmed as hybrids by their null posterior probabilities of belonging to parental classes, were not assigned to a single hybrid class. Nonetheless, sample L82 showed a considerable posterior probability of being a backcross with dog ($q_{BxDog} = 0.721$) and sample L81 had a genotype equally divided by hybrid classes F1 and backcross with dog ($q_{F1} = 0.460$ and $q_{BxDog} = 0.443$, respectively) (Table 3).

Statistical confidence for the assignment of hybrids to specific classes was evaluated using the same Bayesian analysis with simulated genotypes belonging to the six classes (two parental and four hybrid classes). Results revealed that parental individuals were assigned to their correct class with an average posterior probability of 0.997 (the last classified individual in these classes showed a posterior probability of 0.955). For the F1 simulated individuals, a 0.965 average posterior probability was achieved, with only two individuals out of 50 exhibiting posterior probabilities below 0.85. For the F2, an average probability of 0.930 for the assignment of simulated individuals was observed, but six individuals showed probabilities of assignment to this class below 0.85. For simulated backcross with wolf individuals, an average posterior probability of 0.981 was observed and, remarkably, only two individuals had $q_i < 0.875$. For backcross with dog, the results were different: although an average value of 0.857 was achieved for the posterior probability of assignment of simulated individuals to their correct class, a fraction of 28% of

individuals were classified under this value, and the lowest probability obtained for a dog backcross individual that belonged to this class was 0.182. This observed asymmetry in backcross assignment probabilities is certainly related to the higher genetic diversity exhibited by the dog population.

The cases for which simulated individuals were not assigned to their correct class also provide confidence for our results. For example, the posterior probability of assignment to a class was always fractioned among classes. Individuals were never misclassified (i.e. with a probability >0.85) as true parents or as true members of the remaining classes of hybrids.

Direction of hybridization

For the eight hybrid individuals, we investigated both the mitochondrial and the Y-chromosome lineages in order to infer the direction of hybridization (Table 2). To assign our results to wolf or dog mitochondrial genome, we followed the work of Vilà *et al.* (1999), which describes in Iberian wolves the presence of three mitochondrial lineages different from any mtDNA lineage found in dogs. Notably, all eight hybrid individuals in our data show a typical wolf mitochondrial lineage (Table 2), suggesting that female wolves breeding with male dogs is the typical direction of hybridization.

To characterize Y-chromosome lineages in Iberian wolves and dogs, we genotyped all the males in our sample. In 99 male wolves, we found a total of six different Y-linked haplotypes, while in 78 male dogs, we found a total of 14 different Y lineages (data not shown). Five out of the eight hybrid individuals were males and from these, four presented a dog Y-linked haplotype (L81, L318, L352 and L379), while in the remnant case (L58), a typical wolf Y-linked haplotype was observed (Table 2). Only one of these five individuals was assigned to a F1 class (L318), and in this case, a typical dog Y-chromosome haplotype was observed as expected knowing that it presented a wolf mtDNA lineage. For one of the backcrosses with wolf hybrids (L58), a Y-linked wolf lineage was observed, but for the other two backcrosses with wolf hybrids, dog Y-linked lineages were observed, probably meaning that either a male F1 bred with a female wolf or that a female F1 bred with a male dog. For the hybrid male L81, for which it was not possible to assign a hybrid class, a dog Y-linked haplotype was observed.

Discussion

In the last few years, the study of hybridization between wolves and dogs in Europe has evolved from the simple description of anecdotal evidence (Petrucci-Fonseca 1982; Boitani 1983; Vilà & Wayne 1999) to detailed research using cutting-edge molecular and statistical approaches. Examples of the latter were the description of a F1 hybrid in Scandinavia combining the use of maternal, paternal and biparental genetic markers (Vilà *et al.* 2003) or the use of linkage disequilibrium to detect introgressive hybridization in Italian wolves (Verardi *et al.* 2006). In this study, we performed the first large-scale analysis of wolf–dog hybridization in the Iberian Peninsula by combining a set of 42 autosomal microsatellite loci with both mtDNA and Y-chromosome markers to evaluate (i) the differences between Iberian wolves and dogs, (ii) the frequency and geographical distribution of hybridization and (iii) the direction of hybridization.

Differentiation of Iberian wolves and dogs and detection of hybridization

Our analysis of a total of 204 wolves and 196 dogs using a set of 42 autosomal microsatellites revealed the existence of two well-defined clusters (Figs 3 and 4), implying that in Iberia the two entities are well-differentiated and that, despite a long coexistence, introgressive hybridization has apparently not been an important factor shaping both gene pools. Previously, Vilà and colleagues sequenced an mtDNA fragment in more than 100 Iberian wolves and were not able to find a single haplotype typical of dog populations, suggesting that interbreeding between female dogs and male wolves followed by introgression was indeed a rare event (Vilà *et al.* 1997, 1999; Vilà & Wayne 1999). More recently, Ramírez *et al.* (2006) used a set of 13 unlinked microsatellites and did not find any evidence of wolf–dog hybridization in the Iberian Peninsula in a sample of 74 animals. However, our results identified the occurrence of eight wolves (4%) that are probably hybrids. Taken together, this picture is remarkably similar to the one described for Italy, where wolves and dogs form two distinct clusters and the frequency of hybridization appears to be around 5% (Randi & Lucchini 2002; Verardi *et al.* 2006; Randi 2008).

Iberian wolves and dogs also differ in patterns of genetic diversity (Table 1). Specifically, dogs exhibit higher levels of heterozygosity, mean number of alleles and, in particular, a much higher number of private alleles (150 in dogs and 51 in wolves). Precisely the same trends were described by Ramírez *et al.* (2006) for Iberian wolves, and by Randi & Lucchini (2002) for Italian wolves. This evidence additionally supports the sharp distinction between Iberian wolves and dogs, and also the genetic integrity of the wolf gene pool, while patterns in dogs reflect instead possible admixture and a diversity of evolutionary histories. In general, it seems

that the situation of wolf populations in the southern European peninsulas of Iberia and Italy is remarkably similar.

In the present study, we used a total of 42 autosomal microsatellite loci, which is much higher than the 13 microsatellites employed by Ramírez *et al.* (2006) in Iberian wolves, or the 18 unlinked or 16 linked loci used by Randi & Lucchini (2002) and Verardi *et al.* (2006), respectively, when studying Italian wolves. A number of loci higher than those currently applied in most studies (see Koskinen *et al.* 2004 for a general perspective) is absolutely necessary if we want to identify hybrids efficiently. Vähä & Primmer (2006) used computer simulations and convincingly showed that distinguishing between F1 hybrids, backcrosses and parental individuals requires at least 48 loci with an average $F_{st} = 0.21$. While not exactly achieving these numbers, we feel quite confident that our present battery of microsatellites, with an average $F_{st} = 0.16$, has allowed us to draw some firm conclusions.

Frequency and geographical distribution of hybridization

For many years, the magnitude and processes involved in the occurrence of hybridization between wolves and dogs remained essentially unknown and the question persisted as an unsolved concern of conservation biologists (Blanco *et al.* 1992; Boitani 2003). With the development of molecular markers, this issue could be better addressed, and identifying hybrids in natural populations became possible (e.g. Vilà & Wayne 1999; Vilà *et al.* 2003; Randi 2008). However, answering this type of question when two forms are genetically very close is not easy and, consequently, we still lack a general empirical perspective of this problem in wolves. For example, a frequency of hybridization of 0.9% using unlinked microsatellites was initially described in Italy (Randi & Lucchini 2002), but subsequent studies corrected this number to 5% after applying more powerful admixed linkage disequilibrium methods (Verardi *et al.* 2006). In our sample, we found 4% of hybrids, but as a disproportionate effort was devoted to locating putative hybridization events, we cannot know at this time how representative is this frequency for the whole Iberian wolf population. In any case, this frequency is very close to the one detected in Italy, which not only suggests a very similar situation in both peninsulas but also a non-negligible possibility of recurrent gene flow from dogs to wolves. Importantly, this fact additionally reinforces the need to have efficient microsatellite batteries (or other type of markers, such as SNPs) to detect past generation backcrosses.

Our analysis of the geographical distribution of putative hybrid wolves (Fig. 1) suggests that most cases happened in peripheral areas of wolf range, which are presently expanding (Blanco & Cortés 2002; Álvares *et al.* 2005). This is exactly so in northwestern Asturias and south of Salamanca (Castilla y Léon), both in Spain, or Minho, in Portugal. The apparent exception is the sample from Lugo (Galicia, Northwest Spain), but in this case the sampled hybrid wolf was kept in captivity, and although being an animal captured in the wild, we could not be sure about its previous history and accurate origin. Interestingly, the 10 wolves sampled in the isolated and highly endangered population located south of Douro river, in Central Portugal, which is estimated to have less than 10 breeding packs (Álvares *et al.* 2005), showed no evidence of hybridization or introgression with dogs. Verardi *et al.* (2006) described a total of 11 putative hybrid wolves in Italy, which in most cases were also detected in peripheral distribution areas. Our observations thus reinforce previous interpretations considering that in those areas wolves are more prone to cross with free-ranging dogs than in the core distribution area of the species. Interestingly, this situation may have important consequences for the genetic composition of rapidly expanding wolf populations in Europe. In a continent where wolves were extirpated from most regions, advancing populations in the peripheries of the current distribution may be easily introgressed with genetic material from local resident, free-ranging dogs, as has recently been acknowledged from both empirical and simulated data (Excoffier *et al.* 2009). In such a scenario, genetic monitoring of rapidly expanding wolf populations may offer the opportunity of investigating a fascinating but worrying problem from both an evolutionary and conservation standpoint.

Directionality of hybridization

Identifying the directionality of hybridization is essential for a proper understanding of the dynamics of this process in natural populations. In Ethiopian wolves, for example, a substantial modification of the genetic characteristics of the population resulted from hybridization with domestic dogs in which only female wolves mated with male dogs (Gotelli *et al.* 1994). While suggesting that a similar situation could occur in grey wolves, Vilà & Wayne (1999) casted doubts on its impact in the genetic composition of wolf natural populations, because of different biological features of the species (e.g. hybrid integration in packs). Nevertheless, Vilà *et al.* (2003) were able to show compelling evidence of a hybrid wolf resulting from a male dog paired with a female wolf in Scandinavia. Remarkably, all eight hybrid animals described in our study also apparently

result from male dogs crossing with female wolves (Table 2) because in our set of five male and three female hybrids, all possess a typical wolf mtDNA haplotype. Three out of those eight are F1s and other three are backcrosses with wolf, which constitute strong evidence suggesting that hybrids can not only be raised but also be integrated in wolf populations. In fact, field monitoring in the Penouta area (Western Asturias, Spain), where the first hybrids were confirmed in 2004 (L81 and L82), has recorded in subsequent years direct observations of a pack composed of both wolves and dog-like canids, supposedly hybrids (L. Llaneza, unpublished data). The remaining two hybrids are either F2s or backcrosses with dog. Taken together, our results constitute, to our knowledge, the most complete and exhaustively analysed data set ever reported for wolf–dog hybridization and, coupled with previous evidence, strongly suggest that the dynamics of this process in natural populations is mediated by crosses between male dogs and female wolves.

Of special interest is the fact that five of the hybrids come from the same region, in western Asturias, Spain. Sample L64 is a F1 hybrid (from Acebo pack), samples L58 and L352 are backcrosses with wolves (from Bobia pack), and samples L81 and L82 are either F2 or backcrosses (from Penouta pack). In addition, two other individuals included in this study and coming from Penouta pack were identified as pure wolves. In our opinion, this is compelling evidence showing that we were in the presence of a continuum of hybrid classes forming mixed packs that may have started to reproduce just a few generations ago. If this represents a relatively widespread situation in Iberian wolves, especially on the edges of expanding populations, it is possible that additional hybrids may have escaped the detection power of our microsatellite battery and that we have underestimated hybridization. While it is true that present evidence suggests that wolves and dogs remain genetically distinct in Europe, it may be also true that the importance of hybridization in expanding wolf populations may have been underestimated.

In spite of this evidence, the mechanics and dynamics of hybridization are far from being well understood. Assuming that initial crosses involve a female wolf and a male dog, we believe that progenitor dogs may not be feral but hunting or livestock guarding dogs that regularly roam free in wolf habitats. Concerning wolf females, we suggest they are either (i) dispersers in the edge of a marginal and/or expanding population or (ii) solitary individuals resulting from the disruption of a traditional wolf pack because of the loss of the alpha male, severe hunting or the impact of human-related habitat disturbance (e.g. forest fires, large infrastructures). For example, hybrid L318 (Salamanca, Castilla y León, Spain) appeared in 2008, at the border of the wolf range, where a new wolf pack had established in 2003. In subsequent years, that pack was heavily persecuted and finally destroyed to prevent damage to cattle. It is likely that the new hybrid pack formed in 2007, when the last surviving female wolf bred with a male dog. Hybrid packs may exhibit an atypical social structure and different habitat requirements when compared to traditional wolf packs and may be subject to higher pup mortality rates because dogs are unlikely to stay on the pack (as suggested by Vilà & Wayne 1999). Thus, it is possible that hybrid packs are more unstable and constantly emerging and going extinct, a process that is much more likely to occur in marginal areas or low-density wolf populations. However, these and other questions related with the process of wolf–dog hybridization clearly require additional investigation.

Implications for conservation and future research

Our study in the Iberian Peninsula revealed a total of eight (out of 212, or 4%) hybrid wolves of different types and additional evidence suggesting that hybridization can be more prevalent than previously thought. Although hybridization events were only located in peripheral areas of the continuous and expanding wolf range in northwestern Iberia, it would be important to extensively address this subject in isolated and highly endangered wolf populations, such as the ones located in South of Douro river (Central Portugal) and Andalusia (Southern Spain). At this time, it is premature to infer that 4% of the Iberian wolf population are hybrids of some sort. This is because some of the hybrid individuals were not randomly sampled (e.g. L81 and L82 were part of the same pack, or L318 was specifically detected and sampled) and, as mentioned previously, packs that originate hybrids are apparently restricted to marginal areas of wolf distribution. We suggest that the wolf core distribution area is devoid of hybrids, whereas recent dispersers into more disturbed habitats may be more prone to be involved in hybridization events. In contrast, an aspect that may have introduced a different bias in our results, helping to underestimate hybridization, is the way samples were collected. Most samples used in this study were obtained from dead animals and morphologically described as wolves. However, if these animals look like a pure dog, samples would not be collected and still they could be hybrids or, more likely, backcrosses with dog. This possible bias may help to explain why only in the very peculiar situation described in northwestern Asturias, animals were inferred to have a non-negligible probability of being backcrosses with dog. Future studies should try to avoid both types of biases from the beginning.

Independently of the percentage of wolf–dog hybrids now reported in Iberia, the results from this study should be taken with great conservation concern because of the evidence of continuing hybridization. In fact, the possible occurrence of a fairly high number of hybrid individuals in natural populations may have important consequences at both social and political levels. In particular, a strong impact of hybridization with domestic dogs may affect the perception of the wolf as a symbol of untamed nature and wilderness. In order to reduce hybridization between wolves and dogs, we suggest several management actions, of which some are already being implemented and include (i) a regular genetic monitoring of wolf populations, especially in highly humanized regions and marginal areas of the distribution, (ii) population census and genetic survey of dogs occurring within the wolf range, (iii) the sterilization or eradication of known hybrid packs and free-ranging dogs and (iv) studies on the ecology of hybrids focusing on social structure of packs, population dynamics and spatial use.

Finally, one particularly interesting aspect of hybridization between wild species and their domestic counterparts is the possibility of introgressing genetic novelties originated during domestication into natural populations. For example, wolf hybrids frequently show a coat that is more black than normally exhibited by pure wolves, as reported in this study for Iberian wolves (Fig. 2), and also described in Italian wolves (e.g. Randi & Lucchini 2002). Actually, a recent study analysed the evolution of melanism in North American grey wolves and concluded that the phenotypic innovation resulted from a past hybridization event with domestic dogs and rose subsequently in frequency because of natural selection (Anderson *et al.* 2009). Consequently, with hybridization frequencies of about 4–5%, it is tempting to speculate that small and endangered wolf populations in highly humanized southern Europe could find an unexpected source of genetic diversity in mates with free-ranging domestic dogs while maintaining their general gene pool differentiation. This fascinating possibility that may have moulded peculiar biological characteristics of Iberian wolves (e.g. feeding habits, tolerance to humans, low dispersal; Cuesta *et al.* 1991; Blanco *et al.* 2005; Blanco & Cortés 2007), together with other hybridization aspects, can soon be thoroughly investigated with the development of genomic tools, such as the application of the available canine SNP chip.

Acknowledgements

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Data accessibility

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of analyzed microsatellite loci along with repeat motif, allele range, multiplex and reference.

Table S2 Number of alleles per locus (NA), number of private alleles per locus (PA), expected heterozygosity (He) and F_{st} values for each locus in all analyzed samples.

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