

Wolf population genetics in Europe: a systematic review, meta-analysis and suggestions for conservation and management

Maris Hindrikson¹, Jaanus Remm¹, Malgorzata Pilot², Raquel Godinho^{3,4}, Astrid Vik Stronen⁵, Laima Baltrūnaitė⁶, Sylwia D. Czarnomska⁷, Jennifer A. Leonard⁸, Ettore Randi^{5,9}, Carsten Nowak¹⁰, Mikael Åkesson¹¹, José Vicente López-Bao¹², Francisco Álvares³, Luis Llaneza¹³, Jorge Echegaray⁸, Carles Vilà⁸, Janis Ozolins¹⁴, Dainis Rungis¹⁴, Jouni Aspi¹⁵, Ladislav Paule¹⁶, Tomaž Skrbinšek¹⁷ and Urmas Saarma^{1,*}

¹Department of Zoology, Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, 51014 Tartu, Estonia

²School of Life Sciences, University of Lincoln, Green Lane, LN6 7DL Lincoln, UK

³CIBIO/InBio – Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal

⁴Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal

⁵Department of Chemistry and Bioscience, Section of Biology and Environmental Science, Aalborg University, Fredrik Bajers Vej 7H, DK-9220 Aalborg Øst, Denmark

⁶Laboratory of Mammalian Biology, Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania

⁷Mammal Research Institute Polish Academy of Sciences, Waszkiewicza 1, 17-230 Białowieża, Poland

⁸Department of Integrative Ecology, Conservation and Evolutionary Genetics Group, Estación Biológica de Doñana (EBD-CSIC), Avd. Americo Vespucio s/n, 41092 Seville, Spain

⁹Laboratorio di Genetica, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), 40064 Ozzano dell'Emilia, Bologna Italy

¹⁰Conservation Genetics Group, Senckenberg Research Institute and Natural History Museum Frankfurt, Clamecystrasse 12, 63571 Gelnhausen, Germany

¹¹Department of Ecology, Grimsö Wildlife Research Station, Swedish University of Agricultural Sciences, SE-730 91 Riddarhyttan, Sweden

¹²Research Unit of Biodiversity (UO/CSIC/PA), Oviedo University, 33600 Mieres, Spain

¹³ARENA Asesores en Recursos Naturales S.L. c/Perpetuo Socorro, n° 12 Entlo 2B, 27003 Lugo, Spain

¹⁴Latvian State Forest Research Institute “Silava”, Rigas iela 111, LV-2169 Salaspils, Latvia

¹⁵Department of Genetics and Physiology, University of Oulu, 90014 Oulu, Finland

¹⁶Department of Phytology, Faculty of Forestry, Technical University, T.G. Masaryk str. 24, SK-96053 Žvolen, Slovakia

¹⁷Department of Biology, Biotechnical Faculty, University of Ljubljana, Vecna pot 111, 1000 Ljubljana, Slovenia

ABSTRACT

The grey wolf (*Canis lupus*) is an iconic large carnivore that has increasingly been recognized as an apex predator with intrinsic value and a keystone species. However, wolves have also long represented a primary source of human–carnivore conflict, which has led to long-term persecution of wolves, resulting in a significant decrease in their numbers, genetic diversity and gene flow between populations. For more effective protection and management of wolf populations in Europe, robust scientific evidence is crucial. This review serves as an analytical summary of the main findings from wolf population genetic studies in Europe, covering major studies from the ‘pre-genomic era’ and the first insights

* Address for correspondence (Tel: +372 7375099; E-mail: Urmas.Saarma@ut.ee)

of the ‘genomics era’. We analyse, summarize and discuss findings derived from analyses of three compartments of the mammalian genome with different inheritance modes: maternal (mitochondrial DNA), paternal (Y chromosome) and biparental [autosomal microsatellites and single nucleotide polymorphisms (SNPs)]. To describe large-scale trends and patterns of genetic variation in European wolf populations, we conducted a meta-analysis based on the results of previous microsatellite studies and also included new data, covering all 19 European countries for which wolf genetic information is available: Norway, Sweden, Finland, Estonia, Latvia, Lithuania, Poland, Czech Republic, Slovakia, Germany, Belarus, Russia, Italy, Croatia, Bulgaria, Bosnia and Herzegovina, Greece, Spain and Portugal. We compared different indices of genetic diversity in wolf populations and found a significant spatial trend in heterozygosity across Europe from south-west (lowest genetic diversity) to north-east (highest). The range of spatial autocorrelation calculated on the basis of three characteristics of genetic diversity was 650–850 km, suggesting that the genetic diversity of a given wolf population can be influenced by populations up to 850 km away. As an important outcome of this synthesis, we discuss the most pressing issues threatening wolf populations in Europe, highlight important gaps in current knowledge, suggest solutions to overcome these limitations, and provide recommendations for science-based wolf conservation and management at regional and Europe-wide scales.

Key words: *Canis lupus*, conservation genomics, European Union policy, gene flow, large carnivores, wolf management, microsatellites, mitochondrial DNA, SNP, Y chromosome.

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I. INTRODUCTION

The grey wolf *Canis lupus* L. 1758 was historically widespread across Europe, but between the 18th and 20th centuries was progressively eradicated from most of the continent. Once viewed primarily as a threat to public safety, livestock and wild game, the wolf has recently become recognised as an apex predator that plays a key role in ecosystems (Bruskotter, Enzler & Treves, 2011). As a result, conservation measures implemented since the second half of the 20th century and a relaxation of control programs have led to the gradual expansion of many wolf populations in Europe (Musiani, Boitani & Paquet, 2009; Chapron *et al.*, 2014). Currently, approximately 12000 wolves occupy over 800000 km² in 28 European countries, with 9900 individuals present in 22 countries belonging to the European Union (Chapron *et al.*, 2014). In addition, ongoing protection of European wilderness zones, socio-economic changes, innovative laws, public and political commitment, recovery of wild ungulate species and wolf dispersal ability have enabled the species to recolonize many parts of its former range in Europe (Boitani, 1992; Musiani *et al.*, 2009; Musiani, Boitani & Paquet, 2010; Randi, 2011; Chapron *et al.*, 2014; Leonard, 2014; Gilroy, Ordiz & Bischof, 2015; López-Bao *et al.*, 2015) (Fig. 1). Several remarkable examples of wolf recovery in Europe have been described: (i) in Scandinavia, the current population, consisting of 49 family groups and 364–598 individuals (Svensson *et al.*, 2015) was founded by a few eastern (Karelian) immigrants in 1982/83 (Vilà *et al.*, 2003a); (ii) the Italian peninsular population, which in the 1980s occupied only the southern Apennines mountain range, had by the 1990s recolonized the southwestern Alps (Lucchini *et al.*, 2002; Valière *et al.*, 2003; Fabbri *et al.*, 2007, 2014), in the process growing from fewer than 100 individuals in

the 1960s to about 1200–1700 individuals in 2009–2013 (Galaverni *et al.*, 2016) and now coming into contact with the Dinaric-Balkan population in the eastern Alps (Ražen *et al.*, 2016) and expanding to the west to the Pyrenees and Catalonia, Spain (Valière *et al.*, 2003; Lampreave *et al.*, 2011); (iii) the North-west Iberian population has increased since the 1970s (Kaczensky *et al.*, 2013) to between 254 and 322 breeding packs during 1999–2003 (Álvares *et al.*, 2005); (iv) the Central European Lowland population has recently been established in western Poland and eastern Germany *via* recolonization primarily from north-eastern Poland (Czarnomska *et al.*, 2013), with >60 packs established since the first reported reproduction near the German–Polish border in 2000 (Reinhardt *et al.*, 2015). At the same time there are examples of European wolf populations that have recently gone extinct, such as the population in the Alentejo region, southern Portugal, in the 1980s–1990s (Álvares, 2004), or are on the verge of extinction, such as the population in Sierra Morena in southern Spain (López-Bao *et al.*, 2015).

Genetic diversity contributes significantly to the adaptive potential of wolf populations, including the ability to respond adequately to changing environmental conditions and anthropogenic influences, of which climate change, habitat alterations, fluctuations in prey base and emerging infectious diseases are perhaps the most important. In severe cases, loss of genetic diversity through inbreeding can lead to significantly decreased fitness within populations (Reed & Frankham, 2003; Frankham, 2005). This makes evaluation of genetic diversity parameters a particularly important goal in conservation biology (Frankham, 2005; Allendorf, Luikart & Aitken, 2013). On the other hand, the extreme dynamics of population expansion and recolonization exhibited by European wolves is generating a fast-changing distribution at the level of the continent, reflected in the

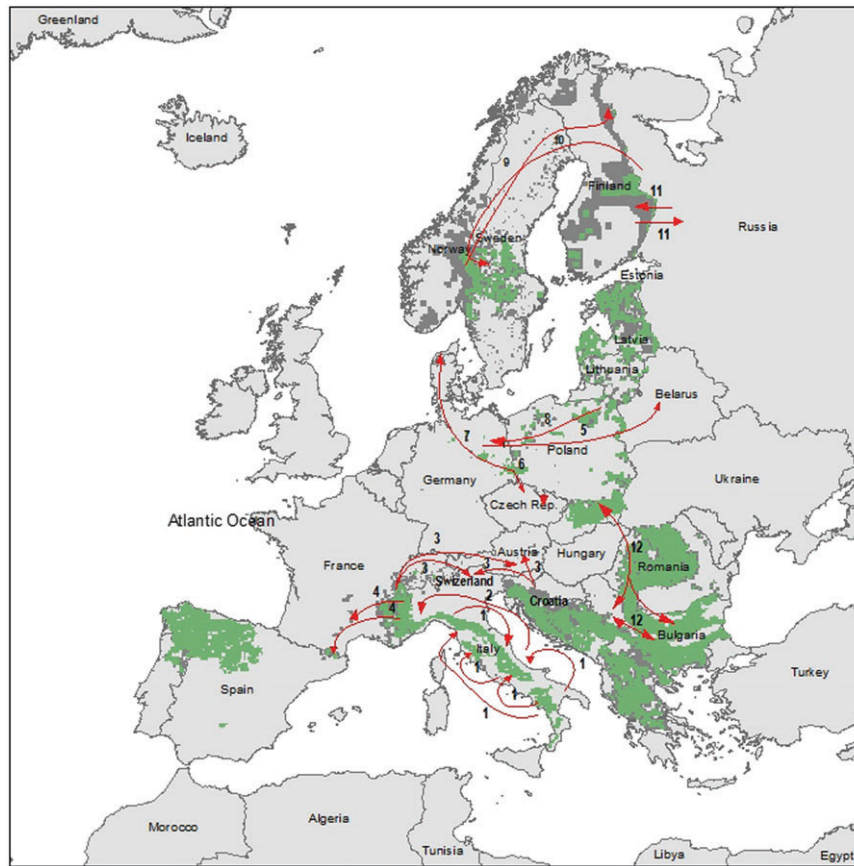


Fig. 1. Wolf distribution and directions of gene flow in Europe. Green indicates wolf permanent occurrence, and dark grey sporadic occurrence (modified from Chapron *et al.*, 2014). Wolf occurrence in Russia, Ukraine and Belarus is not marked on the map. Confirmed dispersal between and within wolf populations is indicated by red arrows (numbers correspond to those given in Appendix S2).

species' landscape genetics (Randi, 2011). While on the one hand the newly established small wolf populations are passing through genetic and demographic bottlenecks with all the accompanying problems (Frankham, 2005; Allendorf *et al.*, 2013), establishment of gene flow between source populations is bringing new opportunities for long-term viability of these emerging populations. Genetic research can provide a deeper understanding of these processes. The approach promises knowledge of both local and large-scale trends in grey wolf genetic composition that is crucial for effective conservation and management of this species and its ecological role throughout Europe.

Here we provide a systematic review of the studies carried out on European wolf populations using genetic markers with different inheritance modes (see Appendix S1).

II. GENETIC PATTERNS OF EUROPEAN WOLF POPULATIONS REVEALED BY DIFFERENT MARKERS

Six main types of genetic markers have been used to study wolves: (i) autosomal microsatellites, (ii) autosomal single

nucleotide polymorphisms (SNPs), (iii) major histocompatibility complex (MHC), (iv) mitochondrial DNA (mtDNA), (v) Y chromosome microsatellites and (vi) Y chromosome SNPs (Figs 2 and 3; note that marker types iii and vi are not shown in these figures). While mtDNA was initially the most frequent choice, autosomal microsatellites quickly gained popularity due to their superior power, compared with mtDNA, for identifying individual animals and for assessing population genetic diversity, population structuring and rates of gene flow between populations. Recently, the depth of population analysis has been increased further by large-scale genome-wide SNP data (vonHoldt *et al.*, 2011; Stronen *et al.*, 2013; Pilot *et al.*, 2014b). As a general rule in mammals, mtDNA is maternally inherited and cannot alone represent all historical and contemporary processes acting upon populations. Nuclear data derived from biparental autosomal loci or from the paternal Y chromosome are thus required to gain a more complete understanding of evolutionary and contemporary population processes of wolves across Europe. Contrary to mtDNA and SNP data that can be combined between studies to cover large areas, the use of microsatellites has an important shortcoming: the data cannot be easily compared between different studies

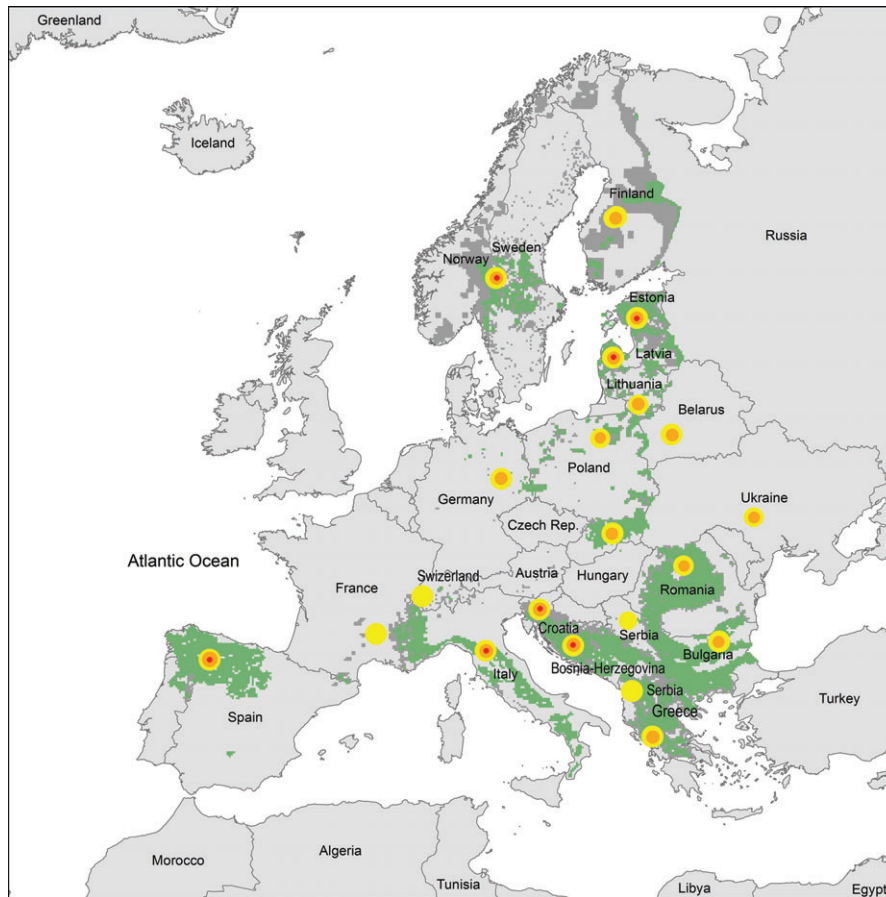


Fig. 2. Different genetic markers used in studies of European wolf populations (see Appendix S1). Yellow: autosomal microsatellites (biparental); orange: mitochondrial DNA (maternal); red: Y-chromosome microsatellites (paternal).

(de Groot *et al.*, 2016). Thus, some microsatellite data sets have been used for specific countries or for a limited region (e.g. Flagstad *et al.*, 2003; Jędrzejewski *et al.*, 2005; Fabbri *et al.*, 2007; Godinho *et al.*, 2011; Jansson *et al.*, 2012, 2014; Hindrikson *et al.*, 2013; Plumer *et al.*, 2016), with few used to characterize wolf populations from wider areas (Pilot *et al.*, 2006, 2014a; Aspi *et al.*, 2009; Sastre *et al.*, 2011; Fabbri *et al.*, 2014) (Fig. 1). The genetic diversity of wolf populations which have suffered demographic bottlenecks and recoveries has also been investigated at the level of loci encoding proteins for the MHC (Seddon & Ellegren, 2004; Arbanasić *et al.*, 2013; Galaverni *et al.*, 2013; Niskanen *et al.*, 2014). In addition to the maternal and biparental markers, investigations of the paternal lineage (Y chromosome) have also become increasingly frequent (Fig. 2), although the majority have focused primarily on wolf–dog hybridization (Sundqvist *et al.*, 2001; Vilà *et al.*, 2003b; Iacolina *et al.*, 2010; Godinho *et al.*, 2011; Hindrikson *et al.*, 2012; Fabbri *et al.*, 2014; Randi *et al.*, 2014).

(1) Biparental markers

(a) Microsatellite loci

Since the early 1990s, when microsatellite markers were first used to study natural populations (Ellegren, 1991),

microsatellites have been the marker of choice for a large number of applications in molecular ecology and conservation genetics. Microsatellite loci, also referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs), are motifs (2–12 base pairs, bp) tandemly repeated in chromosomes, forming blocks of up to 100 bp (Strachan & Read, 1999). Microsatellites are abundant and randomly distributed throughout mammalian genomes, and their rapid evolution and high polymorphism (Roy *et al.*, 1994) together with the large numbers of loci characterised in the domestic dog have made them a useful tool in wolf population studies. Microsatellites have been used to analyse genetic diversity, inbreeding, population structure, gene flow between subpopulations, relatedness of individuals, demography and hybridization with domestic dogs. Nonetheless, a major drawback of microsatellites is the limited comparability of data produced in different laboratories, or even different projects in the same laboratory, requiring careful calibration to overcome the problem. Another drawback has been the lack of a commonly agreed set of microsatellite loci, which makes the direct comparison of results generated in different studies problematic (de Groot *et al.*, 2016). However, with the advent of the genomic era, massively parallel array-based SNP genotyping

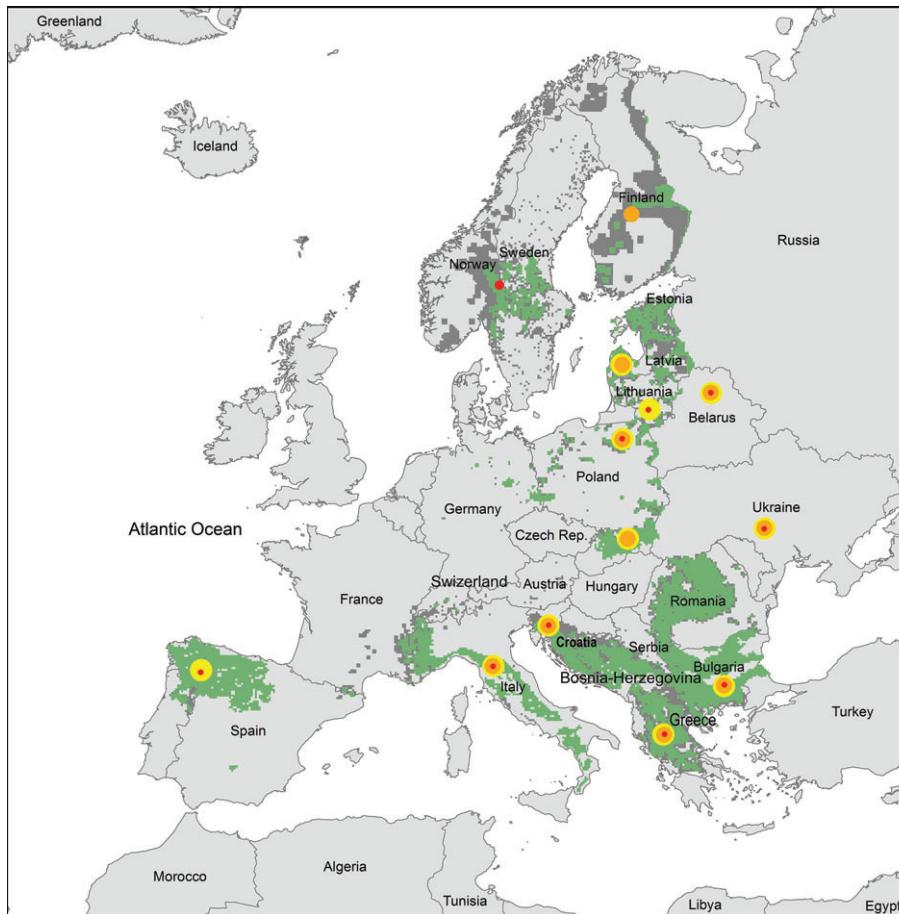


Fig. 3. Wolf autosomal single nucleotide polymorphism (SNP) genotyping studies in Europe. Countries marked in red represent sampling locations from vonHoldt *et al.* (2011; 47,000 SNPs), orange represent sampling locations from Stronen *et al.* (2013; 67,000 SNPs), and yellow represent sampling locations from Pilot *et al.* (2014b; 61,000 SNPs).

and whole-genome sequencing have started to replace microsatellite analysis as the method of choice for many population-level questions.

Results based on microsatellite data have been published for many wolf populations in Europe (Appendix S1; Fig. 2). Below we summarize wolf microsatellite studies at the Europe-wide scale, and for each of the 10 European wolf populations identified by Chapron *et al.* (2014).

(i) *European scale.* Genetic differentiation among European wolf populations is strongly influenced by spatial discontinuities in the wolf range caused by historical persecution by humans. Lucchini, Galov & Randi (2004) showed that the isolated Italian population is strongly differentiated from other European populations, with pairwise fixation index (F_{ST} ; a measure of population differentiation) values ranging between 0.21 and 0.32. They also found the signature of a strong, long-term population decline, suggesting that the Italian wolf population could have been isolated at least for several hundred generations (Lucchini *et al.*, 2004). Sastre *et al.* (2011) also revealed evidence for a dramatic strong bottleneck in recent times in NW Iberian wolves. In these cases, the bottlenecks

have resulted in strong random genetic drift that greatly increased the differentiation between these populations and all other European wolf populations. Significant genetic differentiation has also been observed among the relatively well-connected wolf populations in central and eastern Europe, for example between Baltic and Central European Lowland populations, and Carpathian and Dinaric-Balkan populations. Pilot *et al.* (2006) detected genetic differentiation in central and east European wolves in the absence of obvious physical barriers to dispersal. They suggested that ecological factors, such as climate and habitat conditions, and variations in wolf diet may have influenced gene flow and led to the observed genetic differentiation among wolf populations. This was further supported by the analysis of stable isotope profiles for a subset of genotyped individuals, which provided a quantifiable proxy measure of individual diet and allowed the authors to assess the relationship between individual foraging behaviour and genotype (Pilot *et al.*, 2012). A significant correlation between genetic distance and dietary differentiation was detected even when geographic distance was accounted for as a co-variable, reinforcing the conclusion that dietary preferences and associated habitat choice can

influence the genetic structuring of wolf populations (Pilot *et al.*, 2012). This general mechanism of genetic differentiation detected on a large geographical scale can to some extent also drive local-scale genetic differentiation, and may influence the patterns of recolonization (e.g. Czarnomska *et al.*, 2013; Leonard, 2014).

(ii) *Scandinavian population.* This population consists of about 460 individuals (90% in Sweden, the rest in Norway or in the border area between these countries; Svensson *et al.*, 2015). Exterminated in the 1960s and naturally recolonized since the 1980s by immigrant wolves from Finland (Karelia) (Wabakken *et al.*, 2001; Vilà *et al.*, 2003a), the Scandinavian population is growing and is currently distributed in the central part of Sweden and southeastern Norway. The population has been continuously monitored with genetic methods (Ellegren, Savolainen & Rosen, 1996; Ellegren, 1999; Flagstad *et al.*, 2003; Vilà *et al.*, 2003a,b; Seddon *et al.*, 2005, 2006; Hagenblad *et al.*, 2009). Particular attention has been paid to ongoing immigration from the neighbouring Finnish/Russian (Karelian) wolf population (Flagstad *et al.*, 2003; Vilà *et al.*, 2003a; Seddon *et al.*, 2006), which was shown to coincide with episodes of marked population increase in Russian Karelia (Flagstad *et al.*, 2003), and the identification of four immigrant wolves in northern Sweden in 2002–2005 from Finland (Seddon *et al.*, 2006). The Scandinavian population has been investigated through a period of severe inbreeding depression [inbreeding coefficient (F_{IS}) varied from 0 to 0.42 for wolves born in 1983–2002 in Liberg *et al.*, 2005]; followed by a remarkable genetic recovery thanks to a single immigrant from an eastern (Karelian?) wolf population that brought new genetic material into the population (Vilà *et al.*, 2003a); and through a further period of increasing inbreeding; until the recent immigration of four Finnish/Russian wolves between 2008 and 2013 that rescued the population once again (Åkesson *et al.*, 2016). F_{IS} (hereafter referred to as the inbreeding coefficient) is often misleadingly used synonymously with inbreeding. Inbreeding (and resulting inbreeding depression) is a major conservation concern in several wolf populations. However, the parameter that frequently is reported as ‘inbreeding coefficient’, and often discussed with strong statements about inbreeding in a population (or lack of it), is Wright’s F_{IS} (Wright, 1965). This parameter is easily calculated using genetic data (which probably explains why it is so often reported), but will in most cases carry little information about inbreeding in a natural population. F_{IS} measures departures from Hardy–Weinberg expectations (HWE) in a population, and while it is positive in the case of assortative mating (which does lead to an increase of F), it will be zero in a single generation of random mating. When a population is small, even random mating can lead to matings between relatives, meaning that inbreeding in the population (i.e. F) can be high, but F_{IS} will still be zero or even negative (Waples, 2015). On the other hand, there are common causes for departures from HWE (population substructure, gene flow, genotyping errors) which can increase F_{IS} and cause serious misinterpretations of a population’s inbreeding if the biological meaning of F_{IS} is not understood

correctly. Herein we report inbreeding coefficients estimated in the referenced studies as well as their values, but attempt to avoid some of the biological (mis)interpretations.

(iii) *Karelian population.* The Karelian wolf population is shared between Finland and Russia and consists of 220–245 animals (Natural Resources Institute Finland, 2015). Clear signs of genetic bottlenecks have also been observed in the allele frequency distributions of this population (Jansson *et al.*, 2014). Genetic structure and population processes, including admixture between wolves in the Finnish and Russian parts of the population have been investigated using microsatellites (Aspi *et al.*, 2006, 2009; Jansson *et al.*, 2012). Population size reduction together with the low level of gene flow from the Russian Karelian population (Aspi *et al.*, 2006, 2009) led the Finnish Karelian part of the population into a demographic and genetic crash after 2006, with a significant decline in observed heterozygosity and an increase in inbreeding (Jansson *et al.*, 2012). Compared to the historical Finnish wolf population, almost 20% of microsatellite alleles have not been found in the modern population (Jansson *et al.*, 2014). Although the Karelian wolf population (including Russia) is often seen as a single large management unit, it may consist of smaller units (Aspi *et al.*, 2009; Jansson *et al.*, 2012).

(iv) *Baltic population.* The Baltic wolf population is distributed throughout Estonia (200–260), Latvia (200–400), Lithuania (~300) and north-eastern Poland (270–360), comprising 900–1400 animals in total. As in other parts of Europe, the Baltic wolf population experienced near-extinction in the 1970s and 1980s (Jędrzejewski *et al.*, 2005; Baltrūnaitė, Balčiauskas & Åkesson, 2013), leaving signs of genetic bottlenecks in wolves from Estonia, Latvia (Hindrikson *et al.*, 2013; Plumer *et al.*, 2016) and neighbouring Russia (Sastre *et al.*, 2011). In general, the Baltic population exhibits relatively high levels of heterozygosity compared with many other European wolf populations (Jędrzejewski *et al.*, 2005; Baltrūnaitė *et al.*, 2013; Czarnomska *et al.*, 2013; Hindrikson *et al.*, 2013). Moreover, a cryptic genetic structuring has been found in the Estonian–Latvian part of this population (Hindrikson *et al.*, 2013) and the authors proposed that the four genetic groups identified reflect recent population bottlenecks, severe hunting pressure and immigration. The Estonian population is expanding and has recently (in 2010–2011) recolonized the two largest islands of the country, Saaremaa and Hiiumaa (Plumer *et al.*, 2016).

(v) *Central European Lowland population.* The Central European Lowland population is mainly shared between Poland and Germany (each with >30 packs or 150–200 animals) (Reinhardt *et al.*, 2015), with recent occurrences in Denmark (Andersen *et al.*, 2015), Czech Republic and the Netherlands (Gravendeel *et al.*, 2013), resulting in a total of 300–400 animals ranging over approximately 24000 km². This population was formed in the late 1990s (Andersen *et al.*, 2015), when a small number of wolves from north-eastern Poland (Czarnomska *et al.*, 2013), recolonized the Lusatian border region between Germany and Poland. While the population is expanding steadily (Kaczensky *et al.*, 2013), strong founder effects have likely resulted in genetic

separation between this and the Baltic founder population, despite its close relatedness and evidence for gene flow (Czarnomska *et al.*, 2013; Andersen *et al.*, 2015).

(vi) *Italian peninsular population.* This population is situated along the Apennine Mountains and consists of approximately 321 wolf packs, corresponding to 1212–1711 wolves (Galaverni *et al.*, 2016). Wolves were extirpated from the Alps in the 1920s, and thereafter continued to decline in peninsular Italy until the 1970s, where approximately 100 individuals survived, isolated in two fragmented subpopulations in the central Apennines (Lucchini *et al.*, 2004; Fabbri *et al.*, 2007). Nowadays, the Italian wolf population has a nearly continuous distribution along the Apennines, although three genetic subpopulations (northern Apennines, Central Apennines and Southern Apennines; Fabbri *et al.*, 2007) persist with limited gene flow (Scandura *et al.*, 2011). The Italian wolf population (together with the Scandinavian population) is probably one of the most extensively microsatellite-genotyped wolf populations in Europe (Dolf *et al.*, 2000; Lucchini *et al.*, 2004; Fabbri *et al.*, 2007, 2014; Scandura *et al.*, 2011; Caniglia *et al.*, 2014; Randi *et al.*, 2014). It has been shown that wolves from peninsular Italy have distinct microsatellite allele frequencies that are highly differentiated from other wolf populations typed so far in Europe (Randi *et al.*, 2000; Randi & Lucchini, 2002), except for the Alpine population (Fabbri *et al.*, 2014) and wolves in the Pyrenees in France and Catalonia in Spain (Lampreave *et al.*, 2011; Sastre, 2011), which was established with wolves from Italian origin.

(vii) *Alpine population.* The Alpine wolf population comprises approximately 160 animals and is distributed in the Austrian, French, Italian and Swiss Alps. There are at least 116 animals in France, present in a minimum of 36 wolf permanent presence areas (ONCFS wolf winter survey 2014–2015), 57–89 animals in Italy (Galaverni *et al.*, 2016), eight animals in Switzerland and 2–8 animals in Austria. The Western Alps in Italy, Switzerland and France (Lucchini *et al.*, 2002; Valière *et al.*, 2003; Fabbri *et al.*, 2007, 2014) have been recolonized by Italian wolves, while the eastern and the central Alps are being colonized by wolves from both the Italian and Dinaric-Balkan populations (Fabbri *et al.*, 2014; Ražen *et al.*, 2016). On the other hand, wolves from this population have expanded south-west, recently reaching the French Massif Central and the Pyrenees in 1999 and Catalonia in Spain in 2000, carrying a mtDNA haplotype unique to Italian wolves (W4 in Vilà *et al.*, 1997) (Valière *et al.*, 2003; Lampreave *et al.*, 2011; Sastre, 2011), although without evidence of reproductive success until now, as deduced from the continuous monitoring by the Catalan government.

(viii) *Carpathian population.* The Carpathian population inhabits a large area, including five countries, and consists of ~3000 wolves (2300–2700 in Romania, 340–450 in Slovakia, 250–300 in Poland and a small number of individuals in the Czech Republic and Hungary). The population is largely continuous, although with smaller population fragments (for example in the eastern Czech Republic and Hungary) scattered in the border areas

of the Carpathian population, representing remnants of a previously wider distribution (Boitani, 2000). The Carpathian Mountains represent one of the largest wolf refuge areas in Europe and are regarded as being of particular importance for the long-term survival of the species in Europe because of their size and potential to serve as a link between northern and southern populations (Gula, Hausknecht & Kuehn, 2009). Genetic studies covering the Carpathian wolf population have largely focused on the northern part of the Carpathians in Poland, Slovakia and west Ukraine (Pilot *et al.*, 2006, 2010; Czarnomska *et al.*, 2013; Bakan *et al.*, 2014). Both microsatellite and mtDNA data suggest that the Carpathian wolves are genetically distinct from the neighbouring lowland population (Pilot *et al.*, 2006; Czarnomska *et al.*, 2013) and also from the Dinaric-Balkan population (Bakan *et al.*, 2014).

(ix) *Dinaric-Balkan population.* The Dinaric-Balkan population consists of ~3900 wolves in eight countries: Albania (200–250 individuals), Bulgaria (700–800), Bosnia and Herzegovina (650), Croatia (168–219), Greece (700), Former Yugoslav Republic of Macedonia (466), Serbia (750–850) and Slovenia (32–43) (Chapron *et al.*, 2014). From Slovenia to northern Greece, the wolf range shows substantial continuity along the Dinaric and Balkan Mountains (Musiani *et al.*, 2009; Gomerčić *et al.*, 2010), and Bakan *et al.* (2014) also identified gene flow between Serbia and Bulgaria (Fig. 1; see Appendix S2). Of all European wolf populations, this one spans the largest number of national borders, and is consequently subject to the most diverse array of monitoring and management approaches (Kaczensky *et al.*, 2013). Bulgarian (Lucchini *et al.*, 2004; Bakan *et al.*, 2014; Moura *et al.*, 2014; Pilot *et al.*, 2014a), Greek (Moura *et al.*, 2014), Serbian (Bakan *et al.*, 2014), Croatian (Gomerčić *et al.*, 2010) and Slovenian (Majič-Skrbinšek, 2014) wolves have been studied with microsatellite markers (Appendix S1). Both Bulgarian and Croatian wolves are in the process of recovering from severe bottlenecks that started in the 19th century and lasted up to the 1970s–1980s (Gomerčić *et al.*, 2010; Moura *et al.*, 2014). The Dinaric-Balkan wolf population is a valuable source of genetic diversity for neighbouring populations, as indicated by ongoing recolonization of the eastern and central Alps by Dinaric-Balkan wolves (Fabbri *et al.*, 2014; Ražen *et al.*, 2016), and by the considerable level of gene flow between the Caucasus and the Balkans (Bulgaria) through intermediary populations (Pilot *et al.*, 2014a). The population, however, shows genetic substructuring already at relatively local scales (Fabbri *et al.*, 2014), indicating the need for further research to understand its internal genetic and demographic connectivity and delineate conservation and management units.

(x) *North-west Iberian population.* The North-west Iberian population is shared by Spain and Portugal. The population comprises 63 packs in Portugal (Álvares *et al.*, 2005) and 297 wolf packs in Spain distributed over 91620 km² [MAGRAMA (Spanish Ministry of Agriculture, Food and Environment), 2016]. In a previous census, Blanco, Cuesta & Reig (1990) estimated 294 packs occupying a range of 100000 km². This population ranges across the

north-western region of the Iberian Peninsula and in a small isolated subpopulation south of river Douro in Central Portugal (Álvares, 2004; Blanco, Cortés & Virgós, 2005; MAGRAMA, 2016). At the beginning of the 20th century, Iberian wolves were distributed throughout the peninsula (Rico & Torrente, 2000). However, as in other European wolf populations, in the middle of the 20th century, the Iberian population disappeared from most of its former range and was reduced to an all-time low in the 1970s (Valverde, 1971; Grande del Brío, 1984; Blanco *et al.*, 1990). As a consequence of a severe demographic bottleneck in the 20th century, genetic studies have revealed a low effective population size ($N_E = 43.2-53.8$ in Sastre *et al.*, 2011) and the inbreeding coefficient has varied in this population from 0.153 (Ramirez *et al.*, 2006) to 0.177 (Sastre *et al.*, 2011).

(xi) *Sierra-Morena population.* The Sierra-Morena population is isolated and critically endangered (Blanco & Cortés, 2012; López-Bao *et al.*, 2015), and according to recent Andalusian government reports, no breeding pack was detected in recent years (MAGRAMA, 2016). Ferrand *et al.* (2005) conducted a microsatellite (21 autosomal and 4 Y-chromosome) and mtDNA analysis, but based on a rather small sample size. The authors did not report evidence of hybridization, although this represents an important threat for very small populations (Leonard *et al.*, 2014).

(b) *Single nucleotide polymorphisms (SNPs)*

SNPs represent a widespread source of genetic variation and their abundance throughout the genome makes them highly suitable for population genetic analysis. Whereas earlier studies with microsatellites typically examined <20 markers, SNPs allow simultaneous typing of thousands of loci and thereby increase the statistical power to resolve population structure and processes (e.g. Stronen *et al.*, 2013). In comparison with microsatellites, which have rapid mutation rates per generation (on the order of 10^{-4}), SNPs typically show lower mutation rates ($10^{-8}-10^{-9}$) and simpler mutation patterns that result in relatively low levels of homoplasy (Brumfield *et al.*, 2003). Another great advantage is that SNP data are universally comparable and do not require standardization, while microsatellite data produced in different laboratories have inconsistencies in allele size length that prevent their direct comparison unless meticulous standardization procedures are applied. Moreover, SNPs can potentially provide a better means of genotyping degraded DNA compared to microsatellites (Kraus *et al.*, 2015). On the other hand, microsatellite markers may have some advantages over SNPs, for example in identifying recent events such as new barriers to gene flow or changes in population structure (see Stronen *et al.*, 2013). Using ten microsatellite markers, Aspi *et al.* (2009) showed that Finnish wolves have recently differentiated from Arkhangelsk and Karelian wolves in Russia. However, in a study with 67000 SNPs (Stronen *et al.*, 2013), wolves in Finland appeared well connected to populations in Russia, despite the geographic distance.

Seddon *et al.* (2005) found that 22 out of 24 SNP loci were sufficiently variable in the Scandinavian population to provide a level of accuracy in individual identification equivalent to 12 variable microsatellites. Recently, SNPs have been used in population genetics studies on a regional-scale in the Polish and German (Czarnomska *et al.*, 2013) and Italian (Fabbri *et al.*, 2012) populations, in large-scale European studies (vonHoldt *et al.*, 2011; Stronen *et al.*, 2013; Pilot *et al.*, 2014b), for the identification of wolf-dog hybrids (vonHoldt *et al.*, 2013; Randi *et al.*, 2014; Godinho *et al.*, 2015) and in genetic analysis of non-invasive samples (Valière *et al.*, 2003; Fabbri *et al.*, 2012; Caniglia *et al.*, 2014; Godinho *et al.*, 2015).

Among the large-scale studies, vonHoldt *et al.* (2011) conducted an analysis with the canine SNP genotyping array (47000 SNPs) and found that wolf populations in Italy, Spain, and Eastern/Northern Europe represent distinct units. These results were later supported by a study using 61000 SNPs, where Italian, Iberian and Eastern European (including the Dinaric-Balkan population) wolf clusters were identified (Pilot *et al.*, 2014b). The Italian and Iberian populations had lower heterozygosity and stronger linkage disequilibrium compared to Eastern European populations, indicating that the former have experienced long-term isolation and/or bottlenecks (Pilot *et al.*, 2014b). This study suggested that genetic drift, due to spatial isolation and bottlenecks, is a major evolutionary force behind genetic differentiation of European populations. Moreover, a number of loci showing a signature of diversifying selection were identified, including the loci flanking the platelet-derived growth factor gene, which may influence differences in body size among wolf populations (Pilot *et al.*, 2014b). Stronen *et al.* (2013) evaluated genetic structure in wolf populations from 11 countries (177 wolves, Iberian samples not included) using more than 67000 SNPs and, besides the Italian population, they found the Dinaric-Balkan population and certain clusters in central and northern Europe to be genetically distinct. In a more recent study, Stronen *et al.* (2015) observed differences among north-central Europe, Carpathian Mountains, the Dinaric-Balkan and the Ukrainian Steppe clusters for a number of SNP loci (353 out of 67000 SNPs) and neighbouring genes with known or assumed functions.

A major concern regarding conclusions based on SNPs is that none of the studies have included all European wolf populations. The largest geographical coverage in Europe to date is represented in vonHoldt *et al.* (2011) (Fig. 3), and although Stronen *et al.* (2013) used a larger number of samples per country, several important populations were missing from their analysis. To achieve Europe-wide resolution, significantly improved sampling is required, especially for Alpine, Dinaric-Balkan, Baltic, Karelian (as well as from other areas of Russia), Scandinavian and Iberian populations. Moreover, the combination of data derived from different genotyping platforms can be a challenge. While Illumina's Canine HD chip was used in Stronen *et al.* (2013); vonHoldt *et al.* (2011) and Pilot *et al.* (2014b) used the Affymetrix Canine SNP Genome Mapping Array.

(c) *Major histocompatibility complex (MHC)*

Another way of investigating the genetic diversity of wolves is at the level of loci encoding proteins for the MHC. The MHC is a set of cell surface molecules encoded by a large gene family that controls a major part of the immune system in vertebrates. MHC diversity is shaped by various factors, the most prominent among them being pathogens which are a key selective force in wild animal populations (e.g. Radwan, Biedrzycka & Babik, 2010). High variability in MHC markers may be especially informative in studies of populations that are suspected of having suffered demographic bottlenecks. European wolf populations have maintained relatively high levels of MHC diversity, as shown for Karelian [number of MHC alleles $N = 22\text{--}24$ (Seddon & Ellegren, 2004); $N = 26\text{--}27$ (Niskanen *et al.*, 2014)], Italian peninsular and Alpine populations ($N = 23$; Galaverni *et al.*, 2013), and Dinaric-Balkan population ($N = 31$; Arbanasić *et al.*, 2013). The only known exception is the isolated Scandinavian wolf population, where MHC variation is considerably lower than in other populations ($N = 13$; Seddon & Ellegren, 2004).

(d) *Comparison with studies using bi-parental markers in other large-carnivore populations in Europe, and the main challenges for future investigations*

Microsatellites have also been used to study brown bear (*Ursus arctos*; e.g. Taberlet *et al.*, 1997; Manel *et al.*, 2004; Tammeleht *et al.*, 2010; Kopatz *et al.*, 2012; Straka *et al.*, 2012), Eurasian lynx (*Lynx lynx*; Schmidt *et al.*, 2009; Davoli *et al.*, 2013; Rueness *et al.*, 2014), and wolverine (*Gulo gulo*; Dalerum *et al.*, 2007; Hedmark *et al.*, 2007) in Europe. Even though microsatellites have been proven to be useful markers for studies on all large carnivores, the lack of a common set of universally comparable microsatellite markers between studies has prohibited the analysis of microsatellite data across Europe. Therefore, Europe-wide genetic patterns such as differences in genetic diversity, population structure and connectivity are still missing for all large carnivores.

SNP analyses that have proven to be a way forward in large-scale wolf studies are scarce for other large carnivore species in Europe (Norman, Street & Spong, 2013), largely because of the lack of a closely related domestic species. Rapid advancements in high-throughput and genome-wide sequencing methods are likely to reduce the usage of SNP-chips in the future, depending on the scientific questions asked, and will help to minimize ascertainment bias. Next-generation sequencing (NGS) methods are highly promising since they can provide unbiased data for whole genomes, allowing a wider range of research questions to be addressed, in comparison with SNPs.

However, until whole-genome sequencing becomes considerably cheaper, SNP arrays remain more economical for many purposes, including identification of individuals, detection of wolf–dog hybrids and analysis of population structure and gene flow.

(2) **Uniparental markers**

(a) *Maternal lineage: mitochondrial DNA*

Maternal inheritance, lack of recombination, high mutation rate and high copy-number have made mtDNA an appealing molecular tool in evolutionary biology, conservation genetics and phylogeography for many mammal species, including canids (e.g. Savolainen *et al.*, 2004; Hailer & Leonard, 2008). Non-recombining maternal mtDNA has been widely used not only in phylogeographic studies, but also to study wolf domestication (Vilà *et al.*, 1997; Savolainen *et al.*, 2002; Boyko *et al.*, 2009; Pang *et al.*, 2009; Oskarsson *et al.*, 2012), and wolf–dog hybridization in Scandinavian (Vilà *et al.*, 2003b), Baltic (Andersone *et al.*, 2002; Hindrikson *et al.*, 2012), Italian peninsular (Randi & Lucchini, 2002; Verardi, Lucchini & Randi, 2006; Iacolina *et al.*, 2010; Caniglia *et al.*, 2013; Randi *et al.*, 2014), NW Iberian (Godinho *et al.*, 2011, 2015) and Dinaric-Balkan (Moura *et al.*, 2014) wolf populations.

The hypervariable control region of mtDNA has been sequenced in the majority of studies, either partially (e.g. Vilà & Wayne, 1999; Flagstad *et al.*, 2003; Valière *et al.*, 2003; Ramirez *et al.*, 2006; Seddon *et al.*, 2006; Sastre *et al.*, 2011) or fully (Randi *et al.*, 2000; Lucchini *et al.*, 2004; Hindrikson *et al.*, 2012). Control region sequence data has facilitated the definition of a set of mtDNA haplotypes that differ from dog haplotypes in the majority of European wolf populations. However, the separation is not complete and some haplotypes shared between dogs and wolves have also been found (Vilà *et al.*, 1997, 1999; Randi *et al.*, 2000; Pilot *et al.*, 2010). One of the pioneering mtDNA studies involving both wolves and dogs described ten mtDNA haplotypes in 13 European countries and suggested that European wolves, although restricted to a small fraction of their former range, had been able to preserve a relatively high degree of mtDNA polymorphism (Vilà *et al.*, 1999). Pilot *et al.* (2006) found that wolf populations from Eastern Europe had multiple mtDNA haplotypes that were widely distributed. In a more recent large-scale study, Pilot *et al.* (2010) analysed phylogenetic relationships and geographical distribution of mtDNA haplotypes of 947 contemporary European wolves. They found that haplotypes representing two main haplogroups (1 and 2) overlap geographically, but differ significantly in frequency between populations from southwestern and eastern Europe (see Fig. 1 in Pilot *et al.*, 2010). Haplogroup 1 predominated in Eastern Europe and was fixed in the Iberian Peninsula. These populations shared a common haplotype, suggesting past gene flow *via* extinct intermediate populations from central and western Europe. In the Italian population, haplogroup 2 was fixed and represented by a single haplotype. The unique mtDNA control region haplotype specific to wolves in Italy has neither been found in any other wolf population world-wide (until the Italian wolf population expanded out of Italy in the last decades, moving into the Alps and up to north-eastern Spain), nor in dogs (named as haplotype W4 in Vilà *et al.*, 1997; W14 in Randi *et al.*, 2000 and W22 in Pilot *et al.*, 2010). Low mtDNA variability in wolves has also been found in earlier studies in Iberia (Vilà *et al.*, 1999; Sastre *et al.*, 2011),

suggesting that these peninsular wolf populations in Southern Europe have been isolated for a long time and possibly have lost much of their mitochondrial diversity due to genetic drift, although the possible effect of historic bottlenecks on genetic diversity has not been tested.

Compared with other European populations, wolves in the Dinaric-Balkan population exhibit higher mtDNA control region variability, and the population probably retains a significant proportion of the genetic diversity present in the formerly widespread and continuous European wolf population, as suggested from studies involving Bulgarian (Randi *et al.*, 2000; Moura *et al.*, 2014; Pilot *et al.*, 2014a), Croatian (Gomerčić *et al.*, 2010; Fabbri *et al.*, 2014), and other populations in the Balkans (Pilot *et al.*, 2010; Djan *et al.*, 2014).

In a study comparing the modern and historical Scandinavian population, Vilà *et al.* (2003a) found that the original historical gene pool did not survive the bottleneck and that the present gene pool is made up of new haplotypes brought by founders – immigrants from eastern populations (Finland and north-west Russia). The Finnish part of the Karelian wolf population has experienced a significant reduction in mtDNA haplotype diversity: only three out of eight lineages found in the historic Karelian wolf population before 1920 remained (Jansson *et al.*, 2014).

To date, wolf mtDNA sequences have been characterised from 26 out of the 28 European countries in which the species currently occurs. To analyse genetic relationships between different mtDNA haplotypes across Europe, we found that a 609 bp mtDNA control region fragment provides the best balance between marker size and geographical coverage. Using a median-joining approach (Bandelt, Forster & Röhl, 1999) implemented in program Network 4.510, we constructed a minimum spanning network based on 160 publicly available 609 bp sequences covering all wolf populations in Europe and geographically close populations in West Asia. According to this analysis, European wolves are divided into seven haplogroups (Fig. 4), of which most are of mixed origin, including sequences from several different European wolf populations, although some are more region-specific. The largest haplogroup includes wolves from the Scandinavian and NE-European populations and Greece. Iberian samples were divided between two mixed haplogroups. However, the representation of sequences across Europe is still poor and phylogenetic resolution low due to the relatively short mtDNA sequences (see Appendix S3 for haplotype division).

(i) *mtDNA studies in other large-carnivore populations in Europe and the main challenges for future mtDNA investigations.* MtDNA sequences have also been widely used to study other large carnivore species in Europe: the European lynx (Rueness *et al.*, 2014), wolverine (Zigouris *et al.*, 2013), and especially for the brown bear (e.g. Randi *et al.*, 1994; Taberlet & Bouvet, 1994; Kohn *et al.*, 1995; Saarma & Kojola, 2007; Saarma *et al.*, 2007; Korsten *et al.*, 2009; Davison *et al.*, 2011), including analyses based on complete mitogenomes (Hirata *et al.*, 2013; Keis *et al.*, 2013).

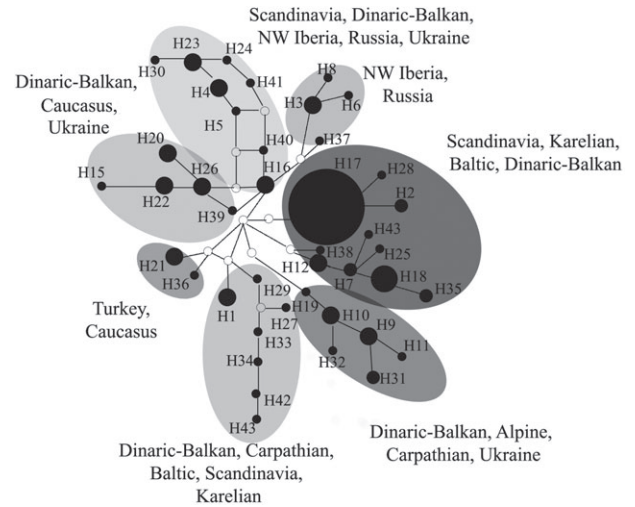


Fig. 4. Median joining network of mitochondrial DNA (mtDNA) control region sequences (609 bp) of 160 wolves from Europe and adjacent populations. Empty circles represent haplotypes not sampled or extinct. Additional data for haplotypes are in Appendix S3.

MtDNA has been and will remain an important genetic marker to study evolutionary processes driven by the female lineages. One of the main drawbacks in wolf mtDNA studies has been the use of short sequences. The analysis of complete mitochondrial genomes of brown bear (Keis *et al.*, 2013) and wolves on a global scale (Koblmüller *et al.*, 2016) clearly demonstrates the advantage of using such data, which revealed spatio-temporal population processes that had not previously been detected using shorter mtDNA sequences. Analysis of genetic diversity and evolutionary trajectories of wolf maternal lineages in Europe is likely to benefit significantly in the future from mitogenome sequencing.

(b) *Paternal lineage: Y chromosome*

Studies using uniparentally inherited Y chromosome loci are scarce compared to biparental markers and mtDNA, primarily due to the shortage of available polymorphic loci. Paternal inheritance and lack of recombination (except in the pseudoautosomal regions) have made the Y chromosome a useful tool for studying uniquely male-inherited lineages, providing an essential complement to maternally inherited mtDNA and biparentally inherited microsatellite or SNP data. When compared with mtDNA, variation in Y-linked loci allows detection of contrasting patterns of male and female population processes (e.g. Bidon *et al.*, 2014). A limited set of paternal Y chromosome microsatellite markers have been used in wolf population genetics to investigate colonization patterns (Sundqvist *et al.*, 2001; Caniglia *et al.*, 2014; Fabbri *et al.*, 2014), population structure and kin relationships (Grewal *et al.*, 2004), hybridization with dogs (Vilà *et al.*, 2003b; Iacolina *et al.*, 2010; Godinho *et al.*, 2011; Hindrikson *et al.*, 2012; Caniglia *et al.*, 2013; Randi *et al.*, 2014) and sex-biased genetic diversity (Sastre *et al.*, 2011).

As with mtDNA, Y chromosome heterogeneity is low in Scandinavian [two haplotypes in Sundqvist *et al.* (2001) and Vilà *et al.* (2003a)] and Iberian [four haplotypes in Sastre *et al.* (2011) and six in Godinho *et al.* (2011)] wolf populations, but significantly higher in western Russia [9–10 haplotypes in Sundqvist *et al.* (2001) and Sastre *et al.* (2011)] and the Balkan region (11 haplotypes in Croatian wolves in Fabbri *et al.*, 2014). In contrast to the pattern of mtDNA variation, Y chromosome variation in Italian wolves is somewhat higher (four haplotypes; Iacolina *et al.*, 2010; Caniglia *et al.*, 2014; Fabbri *et al.*, 2014).

(i) *Y chromosome studies in other large carnivore populations in Europe, and the main challenges for future patrilineal investigations.* Y chromosome investigations are rare in other large carnivores and, besides wolves, have only been used to investigate brown bear populations (Bidon *et al.*, 2014; Schregel *et al.*, 2015). The main drawback of wolf Y chromosome studies is the limited number of polymorphic Y chromosome markers available. Paternal studies would greatly benefit from using a larger number of Y chromosome-specific loci, possibly combining paternal microsatellite and SNP data if neither of them provides sufficient resolution on its own; see for example a study on dingoes (*C. l. dingo*) and dogs by Sacks *et al.* (2013) and a study on humans by Rootsi *et al.* (2013).

III. META-ANALYSIS OF GENETIC VARIABILITY IN EUROPEAN WOLVES

(1) Materials and methods

Meta-analyses of genetic diversity from microsatellites are usually composed of data sets that vary greatly in the identity and number of markers used, and this applies to the data available on European wolves. This complicates the comparison of diversity estimates between studies. One option to overcome this limitation was presented by Skrbinišek *et al.* (2012) who used the reference population approach, scaling the genetic diversity to the genetic diversity of a single well-studied reference population that was used as a calibration ‘yardstick’. By calibrating previously incompatible studies through comparisons with a reference population, they were able to compare the neutral genetic diversity of brown bears from many previously studied populations. However, such a calibration method could not be applied to wolf studies as the number of overlapping loci analysed in different studies is too small (in several instances only three out of 16 loci were identical; Appendix S4, see also de Groot *et al.*, 2016). Nevertheless, as the number of microsatellite loci analysed in different studies is relatively large, we consider the heterozygosity parameters sufficiently robust.

To describe general large-scale trends and patterns of genetic variation in European wolf populations, we analysed the results of previous microsatellite studies and included new data, which altogether covered 10 European wolf populations in 19 countries: Russia, Norway, Sweden, Finland, Estonia, Latvia, Lithuania, Poland, Germany, Czech Republic,

Slovakia, Belarus, Italy, Croatia, Bulgaria, Bosnia and Herzegovina, Greece, Spain and Portugal (Appendix S4; Fig. 5) (note that the Sierra-Morena population is missing due to a lack of data, while data for Russian populations are included). Observed and expected heterozygosities (H_O , H_E), inbreeding coefficient (F_{IS}), allelic richness (R_A) that uses a rarefaction on the minimum number of samples per population in the study, and the number of alleles per locus (N_A) were extracted. If the inbreeding coefficient was presented only for subgroups, it was calculated for the whole population according to the sample-size weighted heterozygosities as $F_{IS} = 1 - H_O/H_E$ (Hartl & Clark, 1997).

Linear trend surface analysis was applied to each variable to determine the presence and direction of a gradient (Fortin & Dale, 2005), followed by a test of the spatial trend. The analysis calculated spatial autocorrelation (SAC) structure via variogram modelling and spatial weighting. We used the R function *gls* with spherical SAC structure in the package *nlme* (Pinheiro *et al.*, 2013) with rotated geographic coordinates along the gradient direction. The Lambert conic conformal coordinate system was used to determine the constant azimuthal direction of the trend over the large area analysed. The coordinate system was rotated around the spatial centre of the sample points and coordinates used for testing the trend’s significance were measured relative to the centre. Due to a relatively small number of data points, we focused on general patterns and did not test non-linear effects, but analysed the pattern in the 10 European populations separately. After the trend surface analysis, the presence of residual spatial autocorrelation was tested using Moran’s autocorrelation index (I) and the compatible test of significance in the R package *ape* (Paradis, Claude & Strimmer, 2004).

(2) Results

(a) Genetic variation of the European wolf populations

We compared four indices of genetic diversity for 10 wolf populations in Europe (Table 1; Fig. 5; Appendix S4). Averaged genetic diversity was lowest in the populations in Iberia and Italy. The Iberian population was also characterised by the lowest allelic richness. The highest heterozygosity was observed in the largest population (Dinaric-Balkan, see Table 1).

None of the variables were correlated with the size of the distribution area of analysed populations. The sample size was negatively correlated with two of the genetic indices (H_E : $r^2 = 0.32$, $P = 0.025$; R_A : $r^2 = 0.31$, $P = 0.03$). However, the observed heterozygosity and inbreeding coefficient had no correlation with sample size (H_O : $r^2 < 0.01$, $P = 0.71$; F_{IS} : $r^2 = 0.02$, $P = 0.70$). Sample size itself had no trend in Europe and was not affected by spatial autocorrelation.

(b) Genetic trends in the European wolf populations

There was a global spatial trend of heterozygosities (H_O and H_E) in the European wolf population. Heterozygosity values

Table 1. Mean values \pm standard deviation for observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}) and allelic richness (R_A) for the four largest European wolf populations

Region	Sample size (N)	H_O	H_E	F_{IS}	R_A
Northern and eastern Europe*	869	0.606 ± 0.095	0.677 ± 0.055	0.123 ± 0.111	6.08 ± 1.25
South-Europe, including:	2448				
Dinaric-Balkan	338	0.658 ± 0.033	0.700 ± 0.027	0.062 ± 0.018	6.10 ± 0.59
Italy (Italian peninsular and Alpine populations)	1622	0.551 ± 0.061	0.568 ± 0.048	0.032 ± 0.036	4.34 ± 0.81
NW Iberia	488	0.526 ± 0.018	0.621 ± 0.021	0.142 ± 0.035	3.76 ± 2.50
Total		0.590 ± 0.085	0.648 ± 0.067	0.098 ± 0.097	5.44 ± 1.53

*Includes Baltic, Scandinavian, Finnish and Russian wolves.



Fig. 5. The geographic location of wolf microsatellite studies included in the meta-analysis. Colours represent populations according to Chapron *et al.* (2014). The Sierra-Morena population is missing due to lack of data; data for Russian populations are included. The numbers in circles represent ID numbers according to Appendix S4.

were considerably higher towards the north-east and lower in south-western populations (Table 2; Fig. 6). The average range of connectedness of populations suggested that the mean size of wolf functional subunits is about 770 km, as indicated by the extent of significance of spatial autocorrelation on trend model residual values of H_O (650 km), H_E (800 km), and F_{IS} (850 km), (Table 2). The reliability of the detected patterns was indicated by zero or near-zero nugget effects of the variogram models. A small nugget effect indicates low variance among independent estimations (different studies) in the same geographic area, and, by extension, a robust

pattern in the observed variable, and good repeatability of measured values. Allelic richness was distributed relatively evenly over Europe, having only a weak signal of spatial pattern and strong nugget effect of the variogram.

(c) Regional patterns

Various genetic diversity patterns were detected within the two large distinct regions of the wolf European range – Southern Europe (NW Iberia, Alps, Italy, Dinaric-Balkan), and north-east Europe (Russia, Karelia, Baltic, Carpathians,

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Table 2. Spatial trends and local autocorrelation pattern of four genetic diversity indices. A spherical variogram model was used. The variogram nugget is measured relative to maximal modelled semivariance. H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient; R_A , allelic richness; $P_{sp\ corrected}$, spatially corrected P -value of global trend; P_{SAC} , P -value of local spatial autocorrelation

Index	Spatial trend				Local pattern			
	Azimuth of gradient (°)	r^2	df _{residuals}	$P_{sp\ corrected}$	Variogram nugget	Variogram range (km)	Moran's I of trend residuals	P_{SAC}
H_O	68	0.23	54	0.011	0.00	650	0.50	< 0.001
H_E	67	0.32	56	0.025	0.17	800	0.40	> 0.001
F_{IS}	117	< 0.01	53	0.695	0.00	850	0.55	> 0.001
R_A	98	0.31	46	0.032	0.50	2835	0.19	0.037

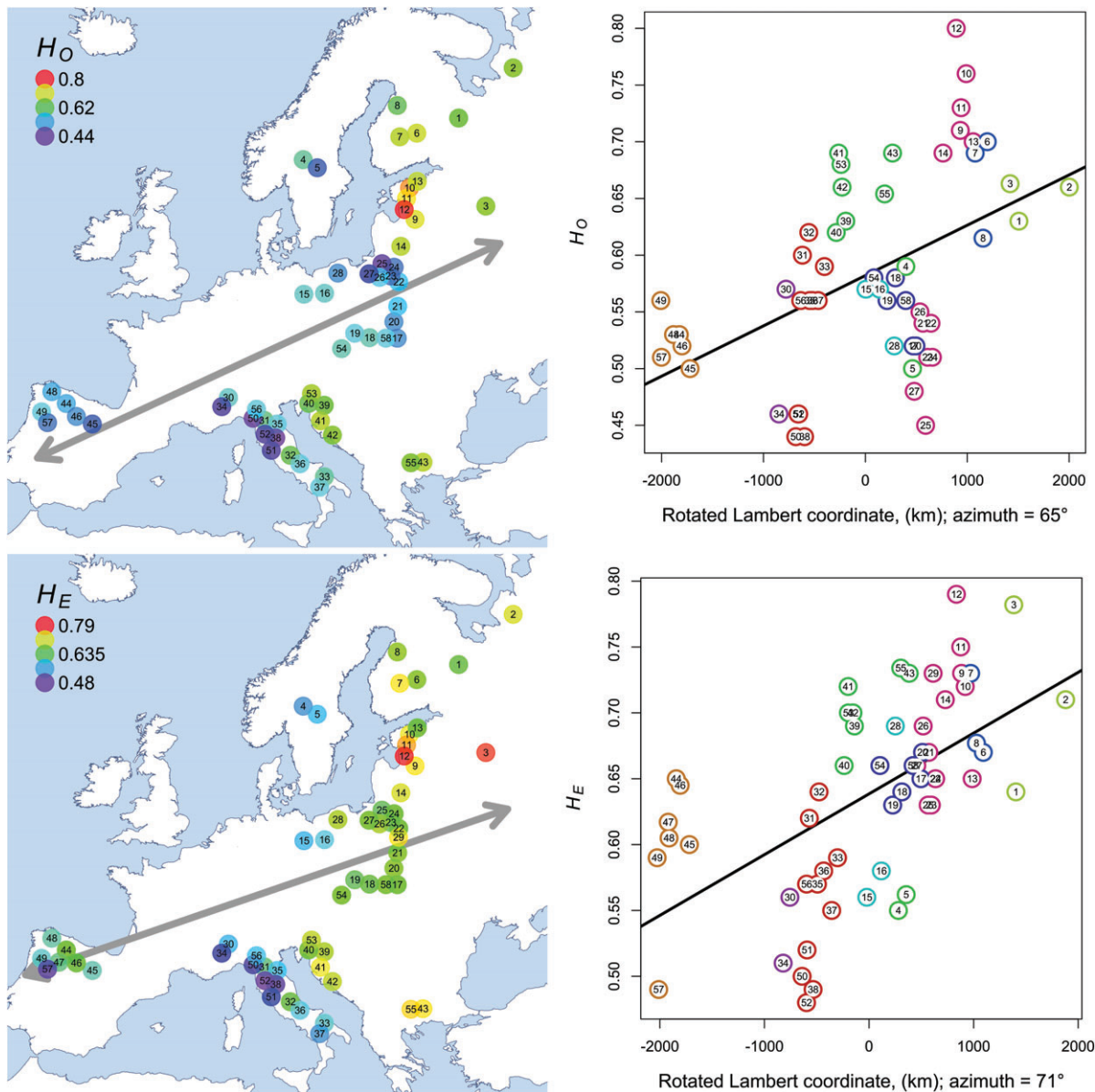


Fig. 6. Spatial trends of observed (H_O) and expected (H_E) heterozygosities in European wolf populations. Significant spatial trends were observed along the slope direction for both H_O and H_E (see Table 2 for trend model parameters). The arrow represents the direction of a gradient (x -axis of the graphs). The numbers correspond to populations according to Appendix S4. Colour codes on the maps (left) correspond to the level of heterozygosity, whereas colours on graphs (right) correspond to populations according to Fig. 5.

Table 3. Spatial trends and local autocorrelation patterns of four diversity indices in two large wolf population clusters. A spherical variogram model was used. The variogram nugget is measured as relative to maximal modelled semivariance. H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient; R_A , allelic richness; $P_{sp\ corrected}$, spatially corrected P -value of global trend; P_{SAC} , P -value of local spatial autocorrelation

Index	Spatial trend				Local pattern			
	Azimuth of gradient (°)	r^2	df _{residuals}	$P_{sp\ corrected}$	Variogram nugget	Variogram range (km)	Moran's I of trend residuals	P_{SAC}
South Europe (NW Iberia, Alpine, Italian peninsula, Dinaric-Balkan)								
H_O	28	0.28	24	0.037	0.33	1200	0.51	0.001
H_E	13	0.14	25	0.031	0.14	1100	0.69	< 0.001
F_{IS}	88	0.07	24	0.909	0.47	1600	0.32	0.034
R_A	40	0.37	17	0.089	0.33	3307	0.32	0.034
North-East Europe (Russia, Karelia, Baltic, Carpathians, Central European Lowland)								
H_O	19	0.38	26	0.225	0.07	800	0.60	< 0.001
H_E	99	0.34	27	0.016	0.55	750	0.16	0.088
F_{IS}	148	0.25	27	0.091	0.33	500	0.35	< 0.001
R_A	19	0.01	25	0.843	0.35	640	0.32	< 0.001

Central European Lowland). In the Scandinavian population, only limited data were available and it was not possible to include this region in the meta-analysis of regional patterns. In southern Europe, a significant gradient of H_O and H_E was directed toward the north-east (Table 3). In the largest continuous population in north-east Europe, a significant west–east gradient of expected heterozygosity (H_E) was observed (Table 3). The lowest values appeared in Germany and the highest in Estonia and Latvia (Fig. 6). The trend model residuals of the H_E were not spatially autocorrelated (Moran's $I = 0.16$, $P = 0.088$). The observed heterozygosity (H_O), inbreeding coefficient (F_{IS}) and allelic richness (R_A) did not exhibit significant spatial trends in north-east Europe, and were significantly autocorrelated in space. The highest genetic variability (H_O and R_A) was found in Estonia and Latvia, and the lowest in Poland. Despite the relatively low heterozygosity in Germany, our results do not indicate significant inbreeding.

IV. WOLF GENETIC VARIATION AND ITS IMPLICATIONS

(1) Genetic variation in European wolf populations

The meta-analysis results are in accordance with recorded population history of wolves in Europe: during the period of demographic decline, larger populations survived in the Balkans and Eastern Europe, while small and fragmented populations remained in the Iberian and Italian peninsulas, and the species was eradicated from central Europe and Scandinavia. Historically, populations in southern Europe have been isolated for long periods of time, possibly for several thousands of years in the case of the Italian (Lucchini *et al.*, 2004; Fabbri *et al.*, 2007) and Iberian (Sastre *et al.*, 2011) populations. Population decline, long-term geographical isolation and a lack of gene flow into the Italian and Iberian wolf populations explain the low genetic diversity and divergence from

other European populations, indicated from microsatellite (Lucchini *et al.*, 2004; Godinho *et al.*, 2011; Sastre *et al.*, 2011), mtDNA (Pilot *et al.*, 2010) and SNP data (vonHoldt *et al.*, 2011; Stronen *et al.*, 2013; Pilot *et al.*, 2014b). Long-term isolation and demographic bottlenecks within these populations have resulted in rather low allelic richness ($R_{A\ Iberian} = 3.8$; $R_{A\ Italian} = 4.3$). Low allelic richness (as a proxy for low overall genetic variability) may compromise the long-term survival of a population, as low genetic variability can become a constraining factor when a population is challenged to adapt to changing environmental conditions. The mean number of alleles per locus in the NW Iberian population is somewhat higher, 4.7–6.4 (Appendix S4), although the isolated subpopulation in central Portugal has a very low estimate of 3.0 alleles per locus. The most effective conservation strategy would require an increase in heterozygosity through elevated gene flow and population growth. The NW Iberian population has been expanding naturally eastward and southward in Spain (Blanco *et al.*, 1990), however in other areas it has disappeared (MAGRAMA, 2016) whereas in Portugal there are no signs of wolf population growth, especially in central Portugal, where the wolf may be on the verge of extinction (Boitani & Ciucci, 2009). Wolves from the Alpine population have reached the Iberian Peninsula in the last decade, but they currently remain in the Eastern Pyrenees and Catalonia, with no connectivity to the NW Iberian wolf population (Valière *et al.*, 2003; Lampreave *et al.*, 2011; Sastre, 2011). Despite the low levels of genetic variability in Italian wolves, this population has active internal gene flow between subpopulations, in large part directed from the Apennines to the Alps (Fabbri *et al.*, 2007). This population has colonized the Alps, forming a new Alpine wolf population that is now coming in contact with wolves of Dinaric-Balkan origin in the east (Fabbri *et al.*, 2014; Ražen *et al.*, 2016), which may result in natural gene flow between the Alpine and Dinaric-Balkan populations in future.

The relatively high heterozygosity in north-eastern populations (Fig. 6) can largely be explained by their

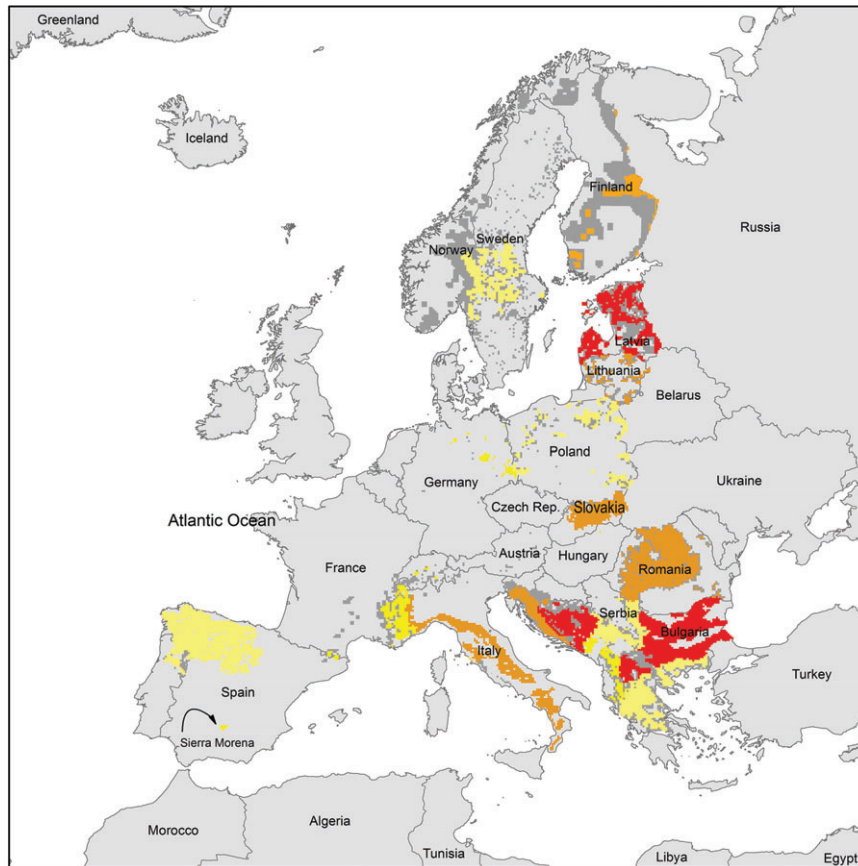


Fig. 7. Levels of legal hunting pressure in European wolf populations. Red, high hunting pressure (>35% of the estimated population size is hunted); orange, medium hunting pressure (10–35%); yellow, low hunting pressure (<10%; including countries where wolves are protected). Note that in Italy and Portugal, where wolf hunting is illegal, the level of hunting pressure comes from poaching that is estimated to remove about 20% and <10% of the total wolf population per year, respectively. For other countries with no official data on poaching available, only legal hunting pressure is illustrated on the map.

demographic connectivity to the large metapopulation in western Russia, which has long served as an important source of immigrants. Due to gene flow between different countries, the Baltic population shows medium to high levels of genetic diversity (Jędrzejewski *et al.*, 2005; Czarnomska *et al.*, 2013; Hindrikson *et al.*, 2013), despite strong hunting pressure (Jędrzejewski *et al.*, 2005; Hindrikson *et al.*, 2013) (Fig. 7). The low H_O (0.45–0.58) and high F_{IS} in Polish and German wolves in the Central European Lowland population (Czarnomska *et al.*, 2013) may indicate inbreeding counterbalanced in a size-limited population by occasional gene flow from the Baltic population, or population structure resulting from high levels of drift in the newly colonized areas. It was suggested that wolves colonizing western Poland and eastern Germany primarily originate from north-eastern Poland (Czarnomska *et al.*, 2013). Despite the relatively high levels of heterozygosity, in our meta-analysis we found signs of inbreeding in north-eastern European wolf populations (Table 1). Recent inbreeding has also been found previously in eastern European wolf populations by Pilot *et al.* (2014b). Inbreeding may increase under strong hunting pressure, which decreases population size and disrupts wolf social

structure (Valdmann, Laanetu & Korsten, 2004; Jędrzejewski *et al.*, 2005; Moura *et al.*, 2014), potentially reducing the quality of traits that define apex predators (Ordiz, Bischof & Swenson, 2013).

(2) Genetic trends in European wolf populations

We found a global spatial trend of heterozygosity with lower values in south-western populations and higher in north-eastern populations (Table 2). Such a trend is probably the result of several factors: recent population demographic history (hunting pressure and bottlenecks), connectivity (isolation in peripheral areas of wolf distribution in Europe) and environmental variables. As environmental gradients in Europe have existed for a long period of time, they most likely have had an impact on genetic variability. For example, it is known that long-term human impact on wildlife in forest habitats has been higher in areas where wolf heterozygosity parameters have low values (for example Iberia and Italy) (Kaplan, Krumhardt & Zimmermann, 2009). The higher levels of heterozygosity in north-eastern Europe may be due to gene flow between northern and eastern European and Russian wolf populations (Pilot *et al.*, 2006; Aspi *et al.*, 2009).

The range of spatial influence (based on analysis of three parameters of genetic diversity) is 650–850 km (Table 2), i.e. the genetic diversity of a wolf population in a certain location is influenced by populations up to 850 km distant. This is, for example, the approximate distance from Tartu (Estonia) to Białowieża (Poland) – indeed, it is likely that the gene flow extends from Estonia to northern Poland as wolves in Europe are known for their long-distance dispersal of 800 km and more (Wabakken *et al.*, 2007; Andersen *et al.*, 2015; Ražen *et al.*, 2016). Despite this, recent findings suggest that gene flow can be restricted even in less urbanised areas, due to prey and habitat specialization (Pilot *et al.*, 2006; Leonard, 2014) and human-built obstacles (Aspi *et al.*, 2009). Radiotracking of wolves has also suggested that few individuals in northern Europe disperse more than 400 km (Kojola *et al.*, 2009) – half of the genetic distance of spatial influence found in our results. Furthermore, few of the dispersal events contribute to gene flow because of human-caused mortality (Kojola *et al.*, 2009; Liberg *et al.*, 2012). These considerations should be taken into account in wildlife management plans dominated by anthropogenic landscapes to avoid significant drawbacks for smaller and more fragmented wolf populations (Delibes, 1990; Hindrikson *et al.*, 2013), particularly in southern regions (Randi, 2011).

V. THE MAIN THREATS TO WOLF POPULATIONS IN EUROPE

(1) Europe in general

Common threats to almost all wolf populations in Europe are overharvesting (including poaching), low public acceptance and conflicts due to livestock depredation (Table 4; Fig. 8), resulting most likely from a lack of knowledge and poor management structure, but also from livestock damage and

deep fears of wolf attacks on humans and dogs. Interactions with domestic dogs leading to disease transfer and/or hybridization have also generated concern (Leonard *et al.*, 2014). However, other threats, such as habitat destruction and large fluctuations in prey base, are also relevant to the majority of populations. Thus, various human-related factors are undoubtedly the main source of threats to wolf populations in Europe, and the generally negative human attitude toward wolves has been and remains the primary threat to wolf populations. Historically, even infectious diseases (e.g. rabies, sarcoptic mange) have not had such a devastating impact on wolf numbers as negative human attitudes, resulting in severe hunting pressure (legal and illegal), which in many areas in Europe led to wolf eradication in the past and continues to threaten small endangered populations (e.g. in Sierra Morena).

Hunting, as well as other direct anthropogenic threats can have genetic consequences (Allendorf *et al.*, 2008), especially for social species such as the wolf (e.g. Creel & Rotella, 2010; Ausband *et al.*, 2015).

The most significant consequence is restriction to gene flow that can result in considerable genetic drift and inbreeding. Severe reduction or loss of population connectivity inside and among European wolf populations is the most challenging factor that requires strong measures, especially in areas where hunting pressure on wolves has been strong for some time (Kaczensky *et al.*, 2013; Jansson *et al.*, 2014; Chapron & Treves, 2016; Plumer *et al.*, 2016). Wolf–dog hybridization is the second most common genetic-related threat in the majority of European wolf populations (Table 4). Hybridization has been shown to increase under strong anthropogenic pressure, especially at the population periphery and in areas with high human-caused mortality (Andersone *et al.*, 2002; Vilà *et al.*, 2003b; Godinho *et al.*, 2011; Hindrikson *et al.*, 2012; Leonard *et al.*, 2014). Another factor that can play an important role in wolf–dog hybridization

Table 4. Common threats to different wolf populations in Europe. y, threat considered important in the population; –, threat not considered important in the population; +/-, threat considered important in some parts of the population; ?, no information

	Alpine	Baltic	Carpathians	Central-European Lowlands	Dinaric-Balkan	Italian Peninsula	NW Karelian	Iberia	Scandinavian	Sierra Morena
Overharvest and poaching	y	y	–	y	y	y	y	y	+/-	y
Low public acceptance	y	y	y	+/-	y	y	y	+/-	y	y
Habitat destruction	–	+/-	–	y	+/-	–	–	y	–	+/-
Barriers to gene flow	–	+/-	–	+/-	+/-	–	+/-	–	y	y
Poor management	–	–	–	–	y	–	y	y	–	y
Poor scientific knowledge	–	+/-	+/-	–	+/-	y	–	y	–	y
Inbreeding	–	+/-	–	+/-	y	–	+/-	y	y	y
Conflicts due to livestock depredation	y	y	y	y	y	y	y	y	y	–
Hybridization with dogs	y	+/-	+/-	+/-	+/-	y	–	+/-	–	y
Prey overharvest	–	–	–	–	+/-	–	–	+/-	–	–
Diseases	–	y	–	+/-	?	–	–	y	+/-	?

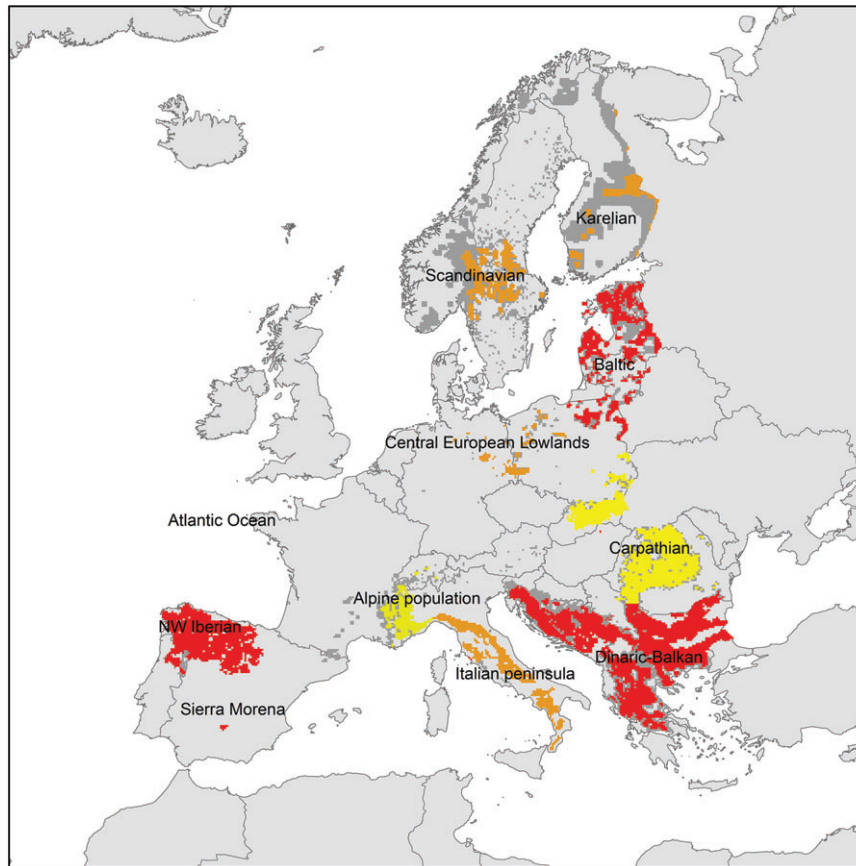


Fig. 8. Threats to wolves in Europe. Threat points are calculated according to Table 4: –, 0 points; ?, 0 points; +/-, 1 point; y, 2 points). Yellow, 1–6 points; orange, 7–12 points; red: 13+ points. Grey cells indicate sporadic occurrence (from Chapron *et al.*, 2014). Wolf occurrence in Russia, Ukraine and Belarus is not marked on the map.

is the disruption of social structure due to high hunting pressure (Valdmann *et al.*, 2004; Jędrzejewski *et al.*, 2005), that can potentially increase the risk of hybridization. Moreover, introgression (following hybridization) can bring selective genetic changes by introducing maladapted genes into wild populations (Leonard *et al.*, 2014).

Large carnivores can coexist with humans if a favourable management policy is applied (Linnell, Salvatori & Boitani, 2008; Treves *et al.*, 2016), but their role as apex predators is reduced if they do not reach ecological functionality (Estes *et al.*, 2011; Ordiz *et al.*, 2013, and references therein). Nevertheless, there is a pressing need to mitigate conflicts in ways that are both effective and acceptable (Sillero-Zubiri & Laurenson, 2001). Two large international legislation systems currently direct wolf management in Europe: the Convention on the Conservation of European Wildlife and Natural Habitats (the Bern Convention), and Council Directive 92/43/EEC on the Conservation of Natural Habitats and of Wild Fauna and Flora (the Habitats Directive; Trouwborst, 2010). Although these international agreements seek to standardize conservation actions across Europe, both the Bern Convention and the Habitats Directive have allowed countries to make national or local modifications to the status of wolves. However, the conservation actions taken to

date have apparently not been sufficient to protect all wolf populations under threat, e.g. in the case of wolf populations in Sierra Morena (see Section V.2i).

To handle the threats and conservation/management issues in European wolf populations in a systematic manner, we first identify the main gaps in current knowledge and suggest solutions to overcome these limitations and then provide suggestions for efficient science-based wolf conservation and management in Europe.

(2) Different populations in Europe

(a) Scandinavian population

By 1966 wolves were functionally extinct on the Scandinavian Peninsula (Wabakken *et al.*, 2001). Since their re-establishment in 1983, wolves in Scandinavia have been subject to long-term monitoring. Due to the very limited number of founders, major conservation issues in this population have been genetic-related: inbreeding depression, low genetic variability and low-level gene flow with other populations (Vilà *et al.*, 2003a; Liberg *et al.*, 2005; Bensch *et al.*, 2006; Råikkönen *et al.*, 2006). Inbreeding has caused strong reductions in two fitness components: winter litter size (Liberg *et al.*, 2005) and recruitment of individuals to breeding (Bensch

et al., 2006). It has also led to a high proportion of congenital malformations in the backbone (Räikkönen *et al.*, 2006). Poaching (Table 4; Fig. 8) has been another major threat, accounting for approximately half of the total mortality in Sweden with more than two-thirds of total poaching remaining undetected by conventional methods (Liberg *et al.*, 2012).

Norway culled some wolves in 2001, claiming the population had already spread too far. In 2010, Sweden licensed the hunting of wolves to keep the population down to 210 individuals, a temporary goal set by the country's parliamentary decree which assumed continuous gene flow from neighbouring populations (although genetic data shows this to be an exceptional rather than a frequent event). The wolf hunt and its effect on conservation and management issues has been highly questioned in popular media and scientific journals (Laikre *et al.*, 2013). The Swedish Society for Nature Conservation has been critical, claiming that culling is against EU legislation as the Swedish wolf population had not reached a healthy status. The issue eventually reached the European Commission (EC): the Union biodiversity legislation requires all member states to follow 'favourable conservation status'. As a result of the complaints, EC sent a reasoned opinion (in June 2015) requesting Sweden to amend its policy to protect the endangered wolf population in the country. This included the request for Sweden to bring wolf hunting into line with EU legislation, thus guaranteeing that the species reach favourable conservation status. A recent report suggests that a long-term goal for the Scandinavian wolf population should be 500 wolves (Kaczensky *et al.*, 2013). Based on another report by commissioned expert statements, the Swedish Environmental Protection Agency decided (in October 2015) that, given that the Scandinavian wolves are a part of a larger northeastern European population by gene flow (including a minimum of one effective immigrant per generation into the Scandinavian population), the Swedish population needs to consist of at least 300 wolves to be considered to have favourable conservation status.

(b) Karelian population

The primary threat to Finnish wolves is illegal killing. The current (Finnish) Karelian population is not only small in size, but also significantly more inbred than previously, and the observed heterozygosity is significantly lower than among wolves born at the end of the 1990s (Jansson *et al.*, 2012). Additionally, gene flow between Russian Karelian and Finnish populations seems to be low (Aspi *et al.*, 2009; Jansson *et al.*, 2012). In order to maintain a genetically healthy and viable wolf population in the long term, the ultimate management goal is to facilitate gene flow between Finnish and Russian parts of the Karelian population (Jansson *et al.*, 2014) and to decrease the hunting pressure. This goal is especially difficult to achieve in the reindeer husbandry area, which is very large (approximately half of Finland), where wolves are eliminated or driven away within days of arrival. The wolf became protected in Finland outside the reindeer husbandry area in 1973, but until 1995 it was listed as a normal game species,

and the population was controlled by hunting (Bisi *et al.*, 2007). Following EU membership in 1995, Finland had to tighten its own legislation concerning the conservation status of the wolf. According to the EC Habitats Directive the wolf is listed in Appendix IV (strictly protected) with an exception in the Finnish reindeer herding area, where the wolf is listed in Appendix V (hunting is possible). The Ministry of Agriculture and Forestry annually grants a restricted number of licenses to kill wolves. The number of animals killed per year (including animals killed in car accidents) has ranged between 5 and 27 during 2000–2005 (Bisi *et al.*, 2007). The Management Plan of the Wolf Population Finland in 2005 (Ministry of Agriculture and Forestry 11b/2005) recommended that Finland should have at least 20 breeding pairs. However, this goal was achieved (during the period 2005–2014) only in 2006, when there were 25 breeding pairs in Finland. A new management plan for wolves was accepted in Finland in 2015 and 'population management' hunting was part of this new plan. Accordingly, the Finnish Wildlife Agency licensed the hunting of 24 wolves in 2015 and an additional 10 wolves can be killed per year in cases of damage or close encounters. The rationale for 'population management' hunting has been hotly debated in Finland.

(c) Baltic population

Low public acceptance due to livestock depredation, especially in islands in western Estonia (Plumer *et al.*, 2016), diseases and human-caused mortality, including illegal killing, are the biggest threats to the Baltic wolf population (Table 4; Figs 7 and 8). However, large infrastructure developments and fragmentation of suitable habitat by intensive forestry and an increase in agricultural land can also pose a significant threat. These problems are expected to remain, if not increase in the future, e.g. the forthcoming construction of Rail Baltic and new highways. Moreover, the new fence currently being built at the Estonian–Russian border will probably decrease gene flow between wolf populations in these countries, although complete isolation is unlikely as wolves can cross Lake Peipus during winter. Although gene flow occurs between Latvia and Estonia (Hindrikson *et al.*, 2013), there is no information on the extent of gene flow for the whole Baltic population. Similarly, there is a lack of knowledge on gene flow with neighbouring populations. Hybridization with dogs has been identified in Latvia, Estonia and northern Poland (Andersone *et al.*, 2002; Hindrikson *et al.*, 2012; Stronen *et al.*, 2013), but not in Lithuania (Baltrūnaitė *et al.*, 2013). However, the rate of introgressive hybridization has not yet been determined; if high, it can pose a threat to wolf long-term adaptive potential (Table 4), or produce a different evolutionary trajectory, towards another kind of canid (possibly well adapted to modified landscapes) and away from the historical ecological role of wolves.

(d) Central European Lowland population

In the expanding Central European Lowland population the main threats are road mortality, high human population

density and illegal killing. In western Poland the loss of any individual has been thought to influence the survival of the pack or interrupt colonization of adjacent areas (Jędrzejewski *et al.*, 2008). Species distribution models have shown that human factors, especially road density and culling might limit the further spread of the species in Germany (Fechter & Storch, 2014) (Table 4). The connectivity of the Central European Lowland population with neighbouring populations is still weak and currently restricted to occasional gene flow from the Baltic population (Kaczensky *et al.*, 2013). However, the population shows a continuous increase, suggesting that the carrying capacity has not been reached yet.

(e) *Italian populations (including both Italian peninsular and Alpine populations)*

The current wolf population expansion on the Italian peninsula is increasing the frequency of conflicts with humans, especially in areas where free-grazing on open pastures is widespread (Meriggi *et al.*, 2011; Milanesi, Meriggi & Merli, 2012). Both Italian peninsular and Alpine populations face threats that are mainly related to low public acceptance, poor management structure, lack of knowledge, persecution and accidental mortality, among others (Table 4; Fig. 8). Of these, illegal killing through poisoning remains the most important cause of mortality (Marucco *et al.*, 2009; Marucco & McIntire, 2010). Hybridization with dogs in areas of the central Apennines has also become a serious concern (Randi, 2008). The genetic diversity of these populations is one of the lowest in Europe (see Table 1) but there are signs of improved connectivity with other European populations: on one hand the Alpine population is incorporating animals from the Dinaric-Balkan population (Fabbri *et al.*, 2014; Ražen *et al.*, 2016); on the other hand, wolves from the Alpine population have expanded south-west, recently reaching the French Massif Central, the Pyrenees and Catalonia in Spain (Valière *et al.*, 2003; Lampreave *et al.*, 2011; Sastre, 2011); however, since there were no wolves in this area, this expansion will not contribute any genetic diversity to the Italian wolf population (see also Fig. 1). In general, administrative fragmentation and the obvious absence of any national authority responsible for wolf management can be considered as important threats that need to be urgently addressed through a renewed effort by the Ministry of Environment, the key agency coordinating the regional governments in implementing national and EU laws.

(f) *Carpathian population*

In Poland, Slovakia and Romania the main problems are connected to livestock depredation (Kaczensky *et al.*, 2013) (Table 4; Fig. 8). For example in Slovakia where depredation on livestock is commonplace, the current overlap of the wolf distribution range with areas devoted to sheep farming is ~90% (Rigg, 2004). In some areas of the Carpathian population range, overhunting and poaching are the main threats (Kaczensky *et al.*, 2013) (Figs 7 and 8). Nevertheless,

the population range and wolf numbers have increased in Slovakia despite hunting during the last 70 years: for example, during the last 20 years the population range has increased by 10% (=1264 km²) (L. Paule, personal communication). There is a general lack of data on gene flow, impact of wolf hunting and hybridization in Ukraine on the number of wolves in neighbouring Poland, Slovakia and Romania.

(g) *Dinaric-Balkan population*

In general, low acceptance (for example in Bulgaria, Slovenia, Bosnia and Herzegovina and The Former Yugoslav Republic of Macedonia) due to pressure on wild ungulate populations and therefore conflicts with hunters (mainly in Greece and Bulgaria) or farmers (livestock conflicts in Bulgaria and Slovenia) are common causes for human persecution (Kaczensky *et al.*, 2013) (Table 4; Figs 7 and 8). In several countries (Serbia, Bosnia and Herzegovina, Bulgaria and The Former Yugoslav Republic of Macedonia) the main threats are limited knowledge on the ecology and population trends of wolves, and poor management structure (Kaczensky *et al.*, 2013) (Table 4; Fig. 8). The population appears to be more or less continuous throughout the Dinaric-Balkan range and is one of the genetically most diverse in Europe (Table 1), having connections with the Alpine (Fabbri *et al.*, 2014) population (Fig. 1). In general, there is a need to clarify the distribution and population sub-structuring within this large population. In some countries such as Albania, Greece and Southern Croatia (Dalmatia), hybridization with dogs might pose a potential risk (Kaczensky *et al.*, 2013; Stronen *et al.*, 2013; Majič-Skrbinšek, 2014). In Bulgaria, a recent genetic study found hybridization of wolves with domestic dogs and possibly also with golden jackals (Moura *et al.*, 2014), while in Greece, an animal with dog ancestry was identified (Stronen *et al.*, 2013).

(h) *NW Iberian population*

This wolf population is considered by the IUCN as 'Near Threatened (NT)' because of the fragmentation in management regimes, the lack of a population-level management plan and the occurrence of largely unpredictable events (human reactions against wolves) that may threaten the population at the local level (IUCN, 2007). In fact, the lack of coordination between authorities in the two countries, together with the separation between science and management (Fernández-Gil *et al.*, 2016), the lack of non-standardized census methods, particularly overestimating wolf pack size (Blanco & Cortés, 2009) and unreliable breeding wolf pack estimations in several regions are considered critical issues for the NW Iberian population, given the fact that wolves are exposed to hunting or to regional administration culls (Echegaray & Vilà, 2010; Fernández-Gil *et al.*, 2016), except in Portugal, where they are fully protected (Pimenta *et al.*, 2005; Kaczensky *et al.*, 2013). Additionally, genetic assessment is not considered for management planning (but see Godinho *et al.*, 2015).

Although there has been an increasing effort to homogenize census methods over recent years (Llaneza, Garcia & Lopez-Bao, 2014; Jiménez *et al.*, 2016), better coordination between different Spanish autonomous regions and between both countries is required.

In Spain two national censuses were carried out in the last 30 years and both suggest similar population and distributional levels: 294 breeding packs ranging over around 100000 km² (Blanco *et al.*, 1990) and 297 packs ranging over 91620 km² (MAGRAMA, 2016). However, several areal shifts occurred between these censuses, including local expansions, declines and extinctions. An important threat is the low acceptance of the species by rural people due to wolf damage to livestock, mass media exacerbation of the conflict pressing the managers and leading to high rates of killings: both legal and illegal in Spain and Portugal (Blanco *et al.*, 1990; Álvares, 2004; Blanco & Cortés, 2009; Fernández-Gil *et al.*, 2016). Other threats include human-related disturbance and loss of habitat quality (non-natural fires, infrastructure development and lack of wild prey, particularly in Portugal (Santos *et al.*, 2007).

In addition, hybridization with dogs is another possible threat in some areas, depending on wolf distribution and human perturbation (Leonard *et al.*, 2014): in a recent genetic survey covering the whole NW Iberian population, 4% of sampled individuals were hybrids (Godinho *et al.*, 2011). On the other hand, genetic heterozygosity (Table 1) is the lowest in Europe and connection with other wolf populations is non-existent, as indicated by the high inbreeding coefficient ($F_{IS} = 0.142$).

(i) Sierra Morena population

The population located in Sierra Morena, southern Spain (Andalusia and Castilla-La Mancha Autonomous Regions) is isolated and critically endangered despite nearly 30 years of legal protection. The population was estimated to contain 6–10 packs in 1988 (Blanco *et al.*, 1990). However, in the following years the breeding population has reduced drastically, perhaps to one pack in recent years (see also López-Bao *et al.*, 2015); although recent estimates confirm the absence of breeding packs in this population (MAGRAMA, 2016). Probable causes are illegal killing to reduce competition for game species and avoid damage to livestock. Unless effective measures likely including population reinforcement are implemented, the Sierra-Morena wolf population will be the first to become extinct in Europe during the 21st century (MAGRAMA, 2016).

VI. SIGNIFICANT GAPS IN KNOWLEDGE AND POSSIBLE SOLUTIONS

Although numerous wolf population genetic and other studies have been published, several significant gaps can be highlighted.

(1) Population coverage

There is a lack of Europe-wide genetic studies covering all European wolf populations. In their recent publication, Chapron *et al.* (2014) divided wolves in Europe into 10 populations, based largely on wolf distribution data. However, for an accurate definition of management units, such information should be coupled with a deeper understanding of wolf dispersal (gene flow) and population genetic structure. Knowledge about levels of gene flow within and between different wolf populations in Europe, and with neighbouring populations in West Asia and countries out of the EU (e.g. in Caucasus, Russia, Belarus, Ukraine and Albania) is limited. However, such knowledge has the potential to identify migration rates and directions to identify possible source/sink populations. It also has the potential to identify unique evolutionary heritages of certain populations (or the lack of it) to identify conservation priorities and guide practical wolf conservation in the future. The best solution would be to create a Europe-wide population genetic project, also engaging researchers from West Asia and non-EU countries.

(2) Sampling protocols

Sampling schemes are not always adequate in terms of sample numbers and geographical coverage. Moreover, analyses are often based on dead individuals (that are not part of the population any longer); ideally, one should be able to obtain a real-time picture of a wolf population, including pedigrees if possible, and track the fate of animals for a longer period of time to understand ongoing population processes, at least in problem areas (Godinho *et al.*, 2015) or in small and isolated populations. To this end, non-invasive sampling (e.g. based on scats) is highly appropriate. The solution is to develop unified sampling protocols and encourage the use of non-invasive sampling methods.

(3) Methodological issues

There is a lack of common methods and sets of genetic markers that are universally comparable between studies. The rapidly developing field of genomics holds great promise for wolf population analysis. However, it is not yet clear which methods will be most appropriate to adopt in terms of data quality and cost. The solution depends also on the research question asked. For analysis of the maternal lineage, the focus in the future should be on sequencing complete mitochondrial genomes, which has already demonstrated its advantages (e.g. Keis *et al.*, 2013; Koblmüller *et al.*, 2016). For the paternal lineage, there is an urgent need to develop a panel consisting of a large number of polymorphic Y chromosome-specific loci (SNPs, microsatellites). For the analysis of autosomal biparental markers, there are three main options: (i) to use (low-coverage) whole-genome sequencing; (ii) to use SNP-chips; or (iii) to use NGS-based microsatellite genotyping. For population analysis, the second and third options are currently more economical, but

the advantages of whole-genome sequencing are apparent: it provides more comprehensive data, enabling coverage of autosomes, the mitogenome and the Y chromosome. The main problem associated with whole-genome sequencing is its economical and analytical cost. If individual identification is required, e.g. for cost-effective and long-term non-invasive genetic monitoring of wolves across Europe, then nanofluidic SNP genotyping technology based on 96 SNP loci (Kraus *et al.*, 2015) and the commercially available multiplex kit for 18 microsatellite loci are perhaps the best options available at present, but the latter requires standardization to compare data produced by different groups. The need for standardization was recently highlighted also by de Groot *et al.* (2016). However, potential conflict can result from the interpretation of management units based on different marker types. Moreover, the NGS methodology revolutionizing conservation genetics may raise questions of how to integrate past microsatellite results with new NGS-produced data. Hopefully it will be possible to integrate results from different marker types and critical thinking, taking into account the quality of the data in the different cases (number of markers, sample size and distribution), will assist in deciding the weight to give to different results.

(4) Hybridization

There is a lack of Europe-wide genetic studies to analyse hybridization between wolves and dogs and the level of introgression of dog genes into wolf populations. It is important to identify wolf populations where introgression can pose a significant threat to population integrity. The critically endangered red wolf (*Canis rufus*) has been the subject of research for several decades and is a good example of how inbreeding and hybridization with coyotes (*C. latrans*) have reduced population viability (e.g. Lockyear *et al.*, 2009; Bohling & Waits, 2011). One solution would be to develop a Europe-wide hybridization project in order to understand the mechanisms that facilitate hybridization and the effects of hybridization on wolf populations, especially in areas with high hunting pressure. All three types of parental markers should be used to monitor hybridization/introgression trends over time, including also their directionality.

(5) Predation

There is limited knowledge of wolf depredation on livestock. Since public attitudes and management measures are largely dependent on rates of wolf depredation on livestock, it is necessary to have accurate measures of depredation rates. As livestock can be killed not only by wolves, but also by domestic dogs and other predators, genetic methods should be used to identify the involvement of wolves and other predator species in livestock depredation (Sundqvist, Ellegren & Vilà, 2008; Echegaray & Vilà, 2010; Caniglia *et al.*, 2013; Milanese *et al.*, 2015; L. Plumer, T. Talvi, P. Männil & U. Saarma, unpublished data). The impact of certain management actions, such as culling, on livestock predation should also be studied carefully, as recent studies

show contrasting results (Wielgus & Peebles, 2014; Bradley *et al.*, 2015; Poudyal, Baral & Asah, 2016); and this type of lethal intervention is highly controversial in modern societies. A solution is to establish a unified genetic methodology to analyse the proportion of livestock killed by wolves, and a unified management reporting system which together may yield scientifically based management recommendations to achieve a goal of decreased attacks on livestock.

(6) Insufficient knowledge of the effects of wolf hunting

Although the effects of hunting are becoming clearer, we still lack a full understanding of its genetic and other consequences (Allendorf *et al.*, 2008). Reduced genetic variation and gene flow, altered population subdivision, disruption of natural social structure, increased hybridization with dogs and reduced survival of pups are known to be among the adverse consequences of hunting on wolf populations (e.g. Valdmann *et al.*, 2004; Jędrzejewski *et al.*, 2005; Creel & Rotella, 2010; Rutledge *et al.*, 2010; Hindrikson *et al.*, 2013; Ausband *et al.*, 2015). However, there is mounting evidence that hunting can also influence ecosystems and human societies in ways that are contrary to expectations or to the intended outcomes: wolf hunting has been found to have undesirable consequences, including ecosystem imbalance, increased livestock predation and increased poaching (e.g. Wielgus & Peebles, 2014; Bradley *et al.*, 2015; Chapron & Treves, 2016; Poudyal *et al.*, 2016). A solution would be to promote scientific investigations on the effects of wolf hunting and provide recommendations for management to decrease undesirable effects of wolf hunting.

VII. SUGGESTIONS FOR SCIENCE-BASED WOLF CONSERVATION AND MANAGEMENT IN EUROPE

For the long-term survival of European wolves and to reach favourable conservation status (mandatory by EU rules), there is a need to increase the overall population size and favour wolf dispersal and connectivity among and within populations. It is therefore important to evaluate the effective size of the entire meta-population to establish scientifically based demographic and genetic targets (Hössjer *et al.*, 2015).

There are several outstanding issues to be solved in order to achieve the most efficient science-based wolf conservation and management (Tables 4 and 5; Fig. 9). Although we focus the discussion on wolves, these issues are generally relevant to all European large carnivores.

(1) Wolf populations should ideally be managed as biological units, i.e. a population should include connected areas with moderate to high gene flow. Further genetic analysis covering all wolf populations in Europe will be necessary to define the exact number and spatial distribution of populations. It is likely that these units will not correspond

Table 5. Priorities and tasks for conservation and management planning of European wolf populations

Priorities	Tasks	Subtasks
Establishment of EU Wolf Scientific Committee: a panel of wolf experts that meets on a regular basis	(1) Consulting officials and managers in EU (2) Coordinating Europe-wide scientific projects on wolf population genetics, wolf-dog hybridization, livestock depredation, food habits, pathogens and public attitudes (3) Raising public awareness	(a) Organise yearly panel meetings (b) Organise biannual wolf conferences (c) Create and maintain European Wolf Webpage (d) Publish scientific papers, annual reports and popular science papers
Establishment of EU Wolf Reference Laboratory (EU-WRL). Establishment of dedicated reference laboratories under EU-WRL: (1) on population genetics (EU-WRL-Gen); (2) on diet and pathogens (EU-WRL-DP)	(1) Conducting Europe-wide scientific analyses (a) EU-WRL-Gen: on population genetics, wolf-dog hybridization, livestock depredation (b) EU-WRL-DP: food habits and pathogens (2) Harmonization of methods (3) Data storage	(a) Establish unified scientific protocols (b) Establish databanks to store wolf data (c) Publish scientific papers, annual reports and popular science papers

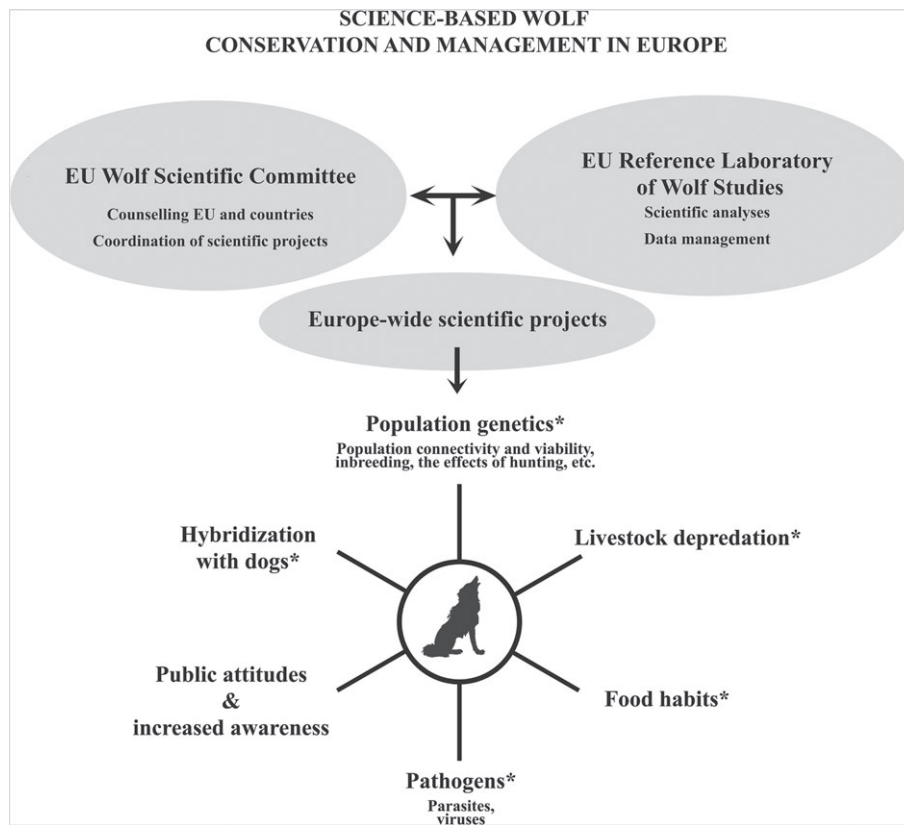


Fig. 9. Science-based wolf conservation and management in Europe, as coordinated by an international scientific committee and reference laboratory. Six major Europe-wide scientific focus areas to promote effective wolf conservation and management in Europe are shown. *, projects that include genetic analysis. See also Tables 4 and 5.

to political boundaries, necessitating communication and ideally, coordination, between governments. Moreover, as some units comprise multiple countries, international cooperation is a key to meaningful management. By contrast, some countries contain multiple units (e.g. Poland). Although units are useful for conservation and management, it may be helpful to underline that (i) natural dispersal between units would be encouraged as an inherent part of wolf biology,

and (ii) units represent evolution-in-progress and are not static.

(2) A European Union Wolf Scientific Committee (EU-WSC), involving scientists from all EU countries with wild wolf populations, should be established as a sub-unit of the European large carnivore scientific committee to guarantee evidence-based scientific decision making. Representatives of government officials, major stakeholders

and scientists from neighbouring wolf-containing countries should also be invited to take part when necessary.

(3) For better implementation of EU legislation and strengthening evidence-based scientific decision making, one option would be to establish a European Union Wolf Reference Laboratory (EU-WRL) (Table 5; Fig. 9). The aim of EU-WRL is to coordinate a network of national laboratories, train laboratory staff and provide reference methods and services to countries without a national reference laboratory. It is important to note that the priority to conduct scientific research would remain with national laboratories; the role of the reference laboratory would be to help develop and adapt reference methods, organise services to those countries without a national laboratory, and coordinate Europe-wide data analysis and data sharing. Thus, EU-WRL would support the creation of a high-performing network of laboratories throughout the European Union, strengthen science-based decision making in wolf conservation and management in the EU, stimulate innovation through the development and adaptation of new methods, tools and standards, and share its knowledge with the Member States, the scientific community and international partners.

Under EU-WRL, several dedicated reference laboratories could be established, responsible for the scientific analyses necessary to provide adequate information on wolf populations across Europe. Two such dedicated reference laboratories are perhaps most urgently required: (i) wolf population genetics (EU-WRL-Gen), and (ii) wolf diet and pathogens (EU-WRL-DP). These laboratories would coordinate genetic, dietary and pathogen research, provide standardization and help in the analysis of samples from different countries in Europe (and beyond). A platform for direct exchange of genetic and other data should be established to facilitate effective information exchange, while guaranteeing intellectual property rights. Studies on food habits provide essential data for the wolf prey-base in different regions in Europe (e.g. Valdmann *et al.*, 2005; Zlatanova *et al.*, 2014) and the ratio of wild prey/livestock in the wolf diet. Knowledge on wolf food habits is crucial for reducing conflicts with various stakeholders and envisaging appropriate conservation-management plans. Although a large number of local studies have been performed, the overall level of knowledge about wolf diet in Europe is poor. Wolf pathogens should also be studied to understand their role in wolf mortality and the potential transmission of pathogens between wolves and free-ranging dogs, and thence to humans. Wolves are well known to transmit rabies, but they can also transmit other hazardous zoonotic pathogens (parasites, viruses, etc.), such as the tapeworms *Echinococcus granulosus* and *E. multilocularis* (e.g. Moks *et al.*, 2006; Marcinkute *et al.*, 2015) that cause the potentially fatal diseases cystic and alveolar echinococcosis, respectively.

(4) Using questionnaires, regular Europe-wide studies should be initiated to investigate public attitudes. Based

on these and other available data, significant effort should be made to improve understanding of the problems and solutions related to wolves and their mitigation. A European Union Wolf Web-page should be established to provide up-to-date information on wolves in Europe (scientific results in popular format, changes in legislation, population data, etc.).

(5) A tradition of biannual European wolf conferences should be established. Such conferences would serve as a main meeting place for wolf experts and other interested parties to present new results and discuss and share ideas to improve wolf research, protection, management, public awareness, etc.

VIII. CONCLUSIONS

(1) Ongoing protection of European wilderness zones, socio-economic changes and recovery of wild ungulates has enabled wolves to recolonize many parts of their former range in Europe. Currently, approximately 12000 wolves occupy over 800000 km² in 28 European countries, with 9900 of these animals present in 22 countries belonging to the European Union. Several remarkable examples of wolf recovery in Europe have been described, e.g. in Scandinavia and Italy. There are also examples of populations that have recently gone extinct, such as in the Alentejo region (Portugal), or are on the verge of extinction, such as in Sierra Morena (Spain).

(2) A Europe-wide meta-analysis was conducted based on the results of available and new microsatellite data. The range of spatial autocorrelation in genetic diversity was 650–850 km, suggesting that the diversity of a given wolf population can be influenced by populations up to 850 km away.

(3) As an important outcome of this synthesis, we discussed the most pressing issues threatening wolf populations in Europe, highlighted important gaps in current knowledge, suggested solutions to overcome these limitations, and provided suggestions for science-based wolf conservation and management at regional and Europe-wide scales. Among these, the most significant are: (i) wolf populations should ideally be managed according to biological units, which requires additional genetic analysis covering all wolf populations in Europe to define the exact number and spatial distribution of populations. (ii) To increase scientific knowledge and inform stakeholders and the general public, there is a need to establish a European Union Wolf Scientific Committee and a European Union Reference Laboratory of Wolf Studies.

(4) If we seek transformative changes in public attitudes and wolf protection/management strategies that result in better coexistence with wolves in human-dominated landscapes, we need Europe-wide scientific knowledge based on the application of unified principles and methods. Science-based knowledge indicates that we need to build management approaches founded on alternatives to wolf

culling, since, in addition to the ethical controversy it attracts in modern society, wolf hunting can result in various undesirable consequences (see also Non-Lethal Wolf Management, 2016).

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XI. SUPPORTING INFORMATION

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

Appendix S1. Different genetic markers used in analyses of the ten European wolf populations.

Appendix S2. Confirmed dispersal between and inside wolf populations in Europe (see also Fig. 1).

Appendix S3. MtDNA haplotypes presented in Fig. 4.

Appendix S4. Wolf populations in Europe included in the meta-analysis.

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