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REVIEW

Decades of population genetic research reveal the need for harmonization of molecular markers: the grey wolf *Canis lupus* as a case study

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ABSTRACT

1. Following protection measures implemented since the 1970s, large carnivores are currently increasing in number and returning to areas from which they were absent for decades or even centuries. Monitoring programmes for these species rely extensively on non-invasive sampling and genotyping. However, attempts to connect results of such studies at larger spatial or temporal scales often suffer from the incompatibility of genetic markers implemented by researchers in different laboratories. This is particularly critical for long-distance dispersers, revealing the need for harmonized monitoring schemes that would enable the understanding of gene flow and dispersal dynamics.
2. Based on a review of genetic studies on grey wolves *Canis lupus* from Europe, we provide an overview of the genetic markers currently in use, and identify opportunities and hurdles for studies based on continent-scale datasets.
3. Our results highlight an urgent need for harmonization of methods to enable transnational research based on data that have already been collected, and to allow these data to be linked to material collected in the future. We suggest timely standardization of newly developed genotyping approaches, and propose that action is directed towards the establishment of shared single nucleotide polymorphism panels, next-generation sequencing of microsatellites, a common reference sample collection and an online database for data exchange.
4. Enhanced cooperation among genetic researchers dealing with large carnivores in consortia would facilitate streamlining of methods, their faster and wider adoption, and production of results at the large spatial scales that ultimately matter for the conservation of these charismatic species.

INTRODUCTION

Large carnivores, such as the grey wolf *Canis lupus*, the Eurasian lynx *Lynx lynx* and the brown bear *Ursus arctos*, were once widespread at continental scales. By the middle of the 20th century, as a result of hunting and persecution, they went extinct from widespread areas. This had important consequences for the structure and function of the ecosystems they once inhabited (Ripple et al. 2014). In recent decades, however, conservation programmes, legal protection, enhanced public awareness and improved habitat conditions resulted in natural re-expansions of the remaining European populations (Chapron et al. 2014), a process that still continues. The comeback of large mammals is not without issues in relation to humans, and long-term coexistence will require active conservation, particularly in the human-dominated landscapes of Europe. A solid understanding of the re-expansion process at the continent level is

needed to support conservation actions, and can be obtained using the tools provided by recent advancements in molecular genetics.

Over the last decades, a large number of genetic markers have been developed to tackle both fundamental and applied research questions, which has increased our understanding of the ecology, the demographic history and the population structure of large carnivores. Recent methodological advances, aimed at surveying more regions of the genome and an ever-increasing number of samples, have allowed researchers to answer long-standing questions with greater levels of detail. Particularly in Europe, the re-expansion of large carnivores is occurring in areas with high densities of people, agricultural land, and urbanized land, and it transcends national borders. Monitoring programmes have been put in place, and genetic analysis of (putative) dispersing individuals between or beyond current populations will be required to assist management and conservation policy in the countries that currently experience re-encounters with wild individuals. Genetic analysis will also yield a valuable opportunity for the detailed study of range expansions, habitat use, dispersal patterns and, in the case of the wolf, hybridization with dogs *Canis familiaris*. Such studies will benefit from the vast amount of knowledge already available, both in terms of methodology and reference data.

While a huge amount of data is already available, incompatibility between the many marker panels currently in use makes even the task of identifying the source population of a long-distance disperser very difficult. In Europe, wild-living wolves, bears and lynxes are present in more than 10 countries (Chapron et al. 2014), a number that is sure to increase in years to come. Each country has its own group or groups of experts, and shapes national conservation policy within the framework of European legislation. In order to set up future studies in the most efficient way and enable the large-scale studies that are required, it will be important to make optimal use of these experiences and to streamline existing methodologies.

Here, we use the wolf as a case study to illustrate the situation. We reviewed the currently available literature on wolf genetics, with a focus on European populations. About 25 years of extensive genetic studies on wolves, mostly in Europe and North America, have yielded a vast number of marker panels and datasets, in what is possibly the most extreme case not only among large carnivores, but also among non-model species in general. This situation is further enhanced by the availability of a plethora of molecular markers and genomic data from the domestic dog, which are usually directly applicable to the wolf. Until now, no systematic review of these methods and their application existed.

The current total number of wolves in Europe (all continental European countries excluding Belarus, Ukraine, and Russia) is estimated to be greater than 12000 individuals (Chapron et al. 2014). The wolf is strictly protected in most countries where it occurs, and the European Union's Habitat Directive lists it as high-priority species (Anonymous 2007). Based on a combination of wolf distribution records and social, ecological, and political factors, the Large Carnivore Initiative for Europe distinguishes 10 populations (Fig. 1): a Sierra Morena (southern Spain), north-western Iberian (northern Spain and Portugal), Alpine (France, Switzerland, Italy), Italian (Apennines, Italy), Dinaric-Balkan (from Slovenia in the north to Bulgaria and Greece in the south), Carpathian (Romania, Czech Republic, Slovakia, Poland, Serbia), central European (western Poland, Germany and Denmark), Baltic (Estonia, Latvia, Lithuania, eastern Poland), Karelian (Finland), and a Scandinavian (Norway and Sweden) population. Numbers per population range from several thousands in the Carpathians and Balkans to a single pack in the Sierra Morena mountains in 2010, in what possibly constitutes the only population of wolves in Europe declining instead of recovering (López-Bao et al. 2015). Throughout Europe, wolves suffer from poaching and negative attitudes of humans towards them, which, together with road kills and habitat fragmentation, can present considerable obstacles for their restoration (Liberg et al. 2011). Even though wolves can disperse over distances of >1000 km (e.g. Wabakken et al. 2007, Ražen et al. 2013), and dispersal distances of >100 km before breeding are common (Linnell et al. 2005), the current genetic status of the European populations seems to be a result of fragmentation and prolonged isolation (Vilà et al. 1999, vonHoldt et al. 2011, Pilot et al. 2014).

In this paper, we present an overview of the genetic methodologies developed and used for wolf research. While our main focus is on wolves in Europe, we make reference to research done worldwide when relevant. We attempt to answer the following questions: 1) Which methodologies are or have been applied to study the genetics of grey wolves, and for which purposes? 2) Are there genetic markers that are commonly used by researchers in many laboratories, and that could be directly used to merge the existing data? 3) How can future research on grey wolves in Europe be better organized to enable optimal use of the available molecular experience and methods, and to provide proper standardization of methods to be applied in the future?

METHODS

We conducted a literature survey in ISI Web of Knowledge in July and November 2014, performing five independent queries using the following search terms: 'wolf AND genetic*', 'wolf AND mtDNA', 'wolf AND microsatellite*',

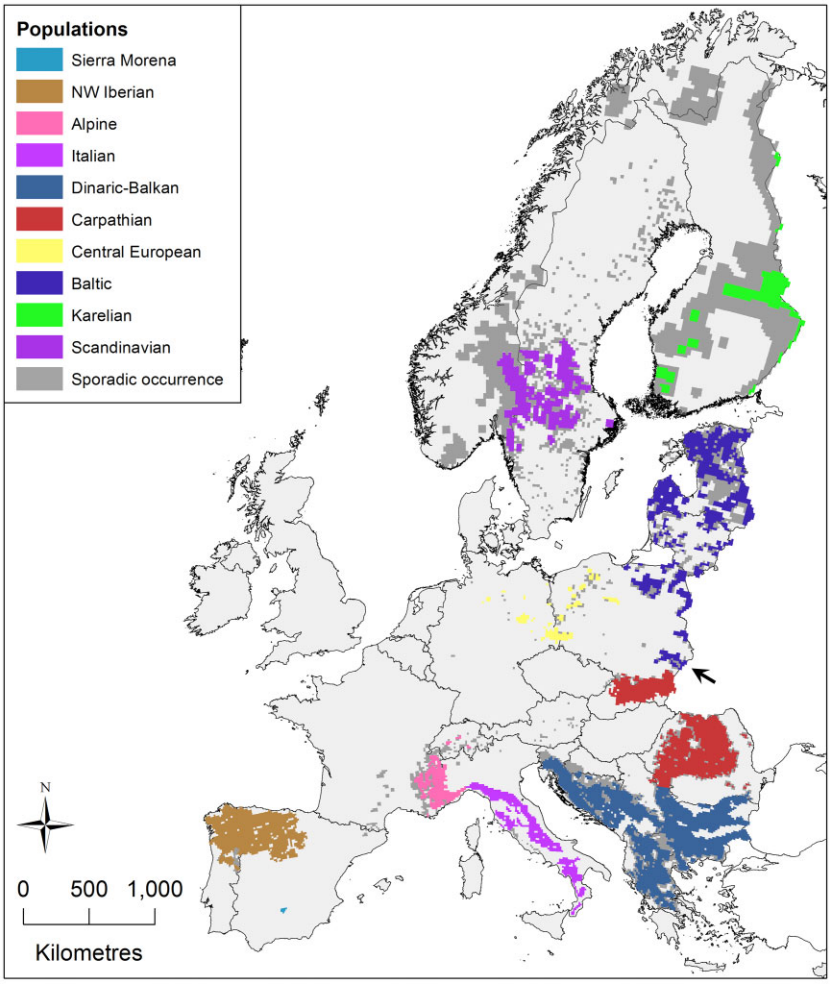

Fig. 1. Distribution of the grey wolf in Europe. Colours indicate permanent wolf presence, and light grey sporadic wolf presence. Excluding Belarus, Ukraine, and Russia, for which no detailed data were available, 10 populations are recognized. Distribution and population divisions are based on Chapron et al. (2014), except for a group in south-eastern Poland indicated with an arrow on the map, which appears here as belonging to the Baltic and not the Carpathian population, following findings by Czarnomska et al. (2013).

'wolf AND SNP*', 'wolf AND MHC'. Each search was refined using the term 'Europe'. Relevant papers that were referenced in the papers we found, that present genetic data for European wolves, but that were missed by these search parameters, were added in the course of reviewing the literature. A total of 81 papers form the basis of our evaluation (Appendix S1).

RESULTS AND DISCUSSION

Research goals for the study of grey wolves in Europe using genetic tools

Genetic methods allow us to deal with a wide range of ecological and management-related questions (Randi 2011), even without visual or physical contact with the study species, through non-invasive sampling (Taberlet et al. 1999). In the case of wolves, these questions can be categorized in three broad groups, which differ in the number of individuals and the spatial scale targeted (Table 1).

Table 1. List of most common types of questions addressed in studies of grey wolves *Canis lupus* in Europe, and the number of papers in which they are addressed

Type of study question	Papers*
Evaluation of individual samples	
Q1: Species identification – is it a wolf?	7
Q2: Individual characterisation / profiling	4
Q3: Population of origin identification	3
Local population monitoring	
Q4: Monitoring genetic parameters of wild or captive-bred populations	24
Q5: Population demography reconstruction	21
Q6: Population substructure determination	10
Q7: Unravelling hybridization patterns between wolves and dogs	12
Landscape-wide patterns	
Q8: Unravelling spatial genetic structure and gene flow among populations	15
Q9: Dispersal pattern reconstruction: changes in distribution areas	9
Q10: Potential identification of ecological discontinuities	2

*Multiple types of questions possible per paper.

IDENTIFICATION OF SINGLE INDIVIDUALS OR PACKS

A large proportion of the applied studies commissioned by local governments or managers deal with species identification (Q1; Table 1). Samples may originate from a wolf-like individual found dead, but are more often scats or hairs, or saliva from bite marks on killed livestock. Recognising wolf presence is a typical question addressed in current recolonization areas, such as north eastern Spain, Denmark, the Netherlands and western Germany, while genetic proof of wolf predation is relevant where this species is established or occurs sporadically, as such proof might result in economic compensation to farmers for livestock losses. In such cases, distinguishing between wolf and dog is valuable (Sundqvist et al. 2008, Caniglia et al. 2013); is it also important to prove cases of illegal killing or poaching (e.g. Savolainen & Lundeberg 1999), or to identify hybrids (Vilà et al. 2003b). Individual identification (Q2) using genetic profiling of samples, such as saliva left on a kill, permits the identification of one or multiple problematic individuals and their sex (Sundqvist et al. 2008, Caniglia et al. 2013, Harms et al. 2015). Especially in recolonization areas, a third type of question in relation to single individuals concerns the assessment of the population of origin (Q3; e.g. Flagstad et al. 2003, Gravendeel et al. 2013, Fabbri et al. 2014). In many instances, the answers to questions about single individuals end up in the grey literature.

MONITORING SINGLE WOLF POPULATIONS

The majority of the papers deal with established and/or endangered populations, mostly in a single country, and are intended to assess population viability and assist management and conservation plans. Such studies may relate to genetic parameters (Q4), such as allelic diversity, functional diversity, effective population size, heterozygosity levels, and/or inbreeding depression (e.g. Liberg et al. 2005). Individual genetic profiles may also be used to assess demographic parameters (Q5), such as local population size or pack numbers and sizes (e.g. Marucco et al. 2009), and may yield the opportunity to build detailed pedigrees, in order to study mating patterns (e.g. Jędrzejewksi et al. 2005). Studies of local population substructure (Q6) may be relevant to define management units or to detect dispersal barriers (e.g. Aspi et al. 2009, Hindrikson et al. 2013). In addition, occasional hybridization with dogs has been reported throughout Europe (Q7; e.g. Randi & Lucchini 2002, Vilà et al. 2003b, Godinho et al. 2011) and may trigger management decisions (Godinho et al. 2015).

GENETIC STRUCTURE ACROSS POPULATIONS

A third type of studies is more broad-scale, concerning genetic diversity, differentiation and gene flow between populations. Following recent advances in high-throughput genotyping, several studies have addressed phylogeographic patterns in large parts of Europe (Q8, e.g. Pilot et al. 2010, 2014, Stronen et al. 2013). Another set of questions (Q9) relates to dispersal routes accounting for past and current range expansions. A number of papers have addressed recolonization of former ranges in, for example, Scandinavia (Flagstad et al. 2003), the Carpathians (Gula et al. 2009) and the Alps (e.g. Valiere et al. 2003, Fabbri et al. 2007). Studies of spatial genetic differentiation and gene flow may also aid in the detection of ecological discontinuities (Q10; Pilot et al. 2006).

The state of the art: genetic methods for wolves developed over the past decades

Molecular tools to study grey wolf populations include markers that are inherited maternally [mitochondrial DNA (mtDNA)], paternally (Y chromosome) and bi-parentally (autosomal and X chromosome). While the majority of studies are based on mtDNA sequences and/or microsatellite markers, assays based on large sets of single nucleotide polymorphisms (SNPs) are becoming increasingly common. We provide an overview of marker application and use to study European populations, and the research institutes that generated the data. We distinguished 11 European wolf populations, comprising the 10 populations as defined by the Large Carnivore Initiative for Europe (Fig. 1, see earlier), augmented with a Russian/Belarussian/Ukrainian group. Research institutes were identified from the materials and methods sections of the papers. If multiple laboratories were involved, these are all mentioned; if no laboratory was mentioned, we assumed that the data are hosted by the first author of the paper. In total, 25 institutes from 14 countries were identified that have generated genetic data on European wolves (Table 2).

MITOCHONDRIAL DNA

Sequence variation in the control region of the mtDNA has been used to study the genetics of wolves, in order to address genetic diversity and phylogeographic history at regional (e.g. Ellegren et al. 1996), continental (e.g. Leonard et al. 2005), and worldwide scales (Wayne et al. 1992, Vilà et al. 1999). The high mutation rate of mtDNA, and in particular, of the hypervariable control region, and its lack of recombination, has offered much resolution in especially phylogeographic studies (although relatively high levels of homoplasy may lead to an underestimation of population differentiation, e.g. Bradman et al. 2011). The fact that mtDNA is relatively easy to amplify made it a popular marker in early phylogeographic studies for modern samples (Vilà et al. 1999), as well as in studies relying on

Table 2. Institutes involved in the generation of genetic data for grey wolves *Canis lupus* in Europe, based on a survey of 81 peer-reviewed papers (Appendix S1), and type of data obtained (mt = mtDNA, SSR = microsatellites, SNP = SNPs, Y = Y-chromosome markers)

Number	Name	Based in	Type of data
1	University of Lausanne (UNIL)	Lausanne, Switzerland	mt, SSR
2	Laboratoire d'Ecologie Alpine (LECA)	Grenoble, France	mt, SSR
3	Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA)	Ozzano dell'Emilia, Italy	mt, SSR, SNP, Y
4	Mammal Research Institute, Polish Academy of Sciences	Bialowieza, Poland	SSR, SNP
5	University of Lincoln	Lincoln, UK	SSR
6	Università degli Studi Gabriele d'Annunzio	Chieti & Pescara, Italy	mt, SSR
7	Centro Gestione e Conservazione Grandi Carnivori	Valdieri, Italy	SSR
8	Senckenberg Research Institute	Gelnhausen, Germany	mt, SSR, Y
9	Aarhus University	Aarhus, Denmark	SNP
10	Estación Biológica de Doñana (EBD-CSIC)	Sevilla, Spain	mt, SSR, Y
11	CIBIO, University of Porto	Vairão, Portugal	mt, SSR, Y
12	Swedish University of Agricultural Sciences (SLU)	Grimsö, Sweden	mt, SSR
13	Uppsala University	Uppsala, Sweden	mt, SSR, SNP, Y
14	University of Zagreb	Zagreb, Croatia	mt
15	University of Oulu	Oulu, Finland	mt, SSR
16	Technische Universität München	München, Germany	SSR
17	Museum and Institute of Zoology, Polish Acadamy of Sciences	Warsaw, Poland	mt, SSR
18	University of Tartu	Tartu, Estonia	mt, SSR, Y
19	University of Sassari	Sassari, Italy	SSR, Y
20	Royal Institute of Technology (KTH)	Stockholm, Sweden	mt
21	Universitat Autònoma de Barcelona	Barcelona, Spain	mt, SSR, Y
22	University of California Los Angeles	Los Angeles, United States	Mt, SNP
23	Lund University	Lund, Sweden	SSR
24	University of Florence	Florence, Italy	SSR
25	University of Bern	Bern, Switzerland	SSR

non-invasive sampling (Valiere et al. 2003) or on museum samples (Leonard et al. 2005). However, its maternal inheritance gives a biased view of population history. For this reason, the information provided by this marker has been complemented with biparentally inherited and patrilineal Y-chromosome markers (microsatellites and SNPs).

Sequenced-based data can easily be compared between studies and laboratories, as they are not platform-dependent (unlike data from microsatellites, see later). The open online *Genbank* repository hosted by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) currently hosts mtDNA sequences of >1000 wolves from multiple European populations. However, at least 11 different primer pairs have been used that do not yield completely overlapping fragments (Appendix S2). Nevertheless, there are a minimum of 230-base pair (bp) overlapping sequences from the control region corresponding to 947 contemporary European wolves from 23 countries (in December 2014) which, together with additional individuals from Asia and North America, make a total of 75 haplotypes (Pilot et al. 2010). In addition, 661-bp sequences from 42 selected contemporary individuals originating from nine European populations revealed 33 haplotypes (e.g. Pilot et al. 2010). New unique haplotypes are still being found (Jansson et al. 2014). At present, an increasing number of papers rely on complete mtDNA genomes (Thalmann et al. 2013).

Y CHROMOSOME AND SEX DETERMINATION

Y-chromosome markers provide a view of the patrilineal evolutionary history. The MS34A/B and MS41A/B markers (Sundqvist et al. 2001, Flagstad et al. 2003) have so far been used most widely (Appendix S2). For example, the number of male founders of the recently re-established Scandinavian population could be determined (Sundqvist et al. 2001), and it could be ascertained that long distance dispersal from Finland to Scandinavia was male-biased (Flagstad et al. 2003). Adding Y-chromosome markers has contributed towards providing a more accurate view of hybridization, including hybrid category and origin (e.g. Vilà et al. 2003b; Godinho et al. 2011; Iacolina et al. 2010).

Amplification of loci both on the X and Y chromosomes allows the sex of individuals to be determined. The method described by Shaw et al. (2003) to sex a number of mammalian species has been applied to Italian wolves (Lucchini et al. 2002), and was further optimized by Seddon et al. (2005) for non-invasive sampling of wolf faeces.

MICROSATELLITES

Microsatellite-based genetic profiling has become a key element in monitoring programmes (e.g. Caniglia et al. 2014), and it is now routinely used to obtain reliable individual profiles from non-invasively collected samples. Such samples require special attention due to associated problems of allelic dropout and false alleles (e.g. Taberlet et al. 1996, Harms et al. 2015), and thus require the implementation of a multiple-tube approach that allows genotyping errors to be detected and corrected for, and quality indexes and maximum-likelihood genotype reliability to be estimated (Miller et al. 2002, Miquel et al. 2006). Microsatellite profiles have allowed the recognition of differentiation in European populations (e.g. Pilot et al. 2006), the performance of assignment tests to determine the most likely population of origin of a dispersing individual (Vilà et al. 2003a), the estimation of levels of gene flow among existing populations (Aspi et al. 2006), and the deciphering of colonization routes during current range expansions (Fabbri et al. 2007). Genetic capture-recapture population size estimates have been possible (Marucco et al. 2009), as well as estimates of effective population size (Aspi et al. 2006), and detection of past fragmentation and bottlenecks (e.g. Lucchini et al. 2004). Reconstruction of pedigrees is now feasible, and has yielded interesting insights on kinship relationships and pack composition of recolonizing populations, both in Europe and North America (Liberg et al. 2005, vonHoldt et al. 2008). Microsatellites have also been popular markers for the detection of hybridization throughout Europe (e.g. Godinho et al. 2011, Hindrikson et al. 2012, Randi et al. 2014).

An important drawback of traditional microsatellite analyses, however, is that it is not possible to compare microsatellite data directly between laboratories. Allele sizes are platform-dependent, peak scoring relies on expert, but subjective evaluation, and different primers might be used to amplify the same locus, all resulting in different size definitions of the same allele by different researchers. Thus, combination of datasets produced by different laboratories is challenging and requires calibration through the exchange of samples with known profiles, which in practice complicates studies. Adoption of a common allele nomenclature is also necessary.

Large numbers of canine microsatellite loci (>30000) have been identified (e.g. Wong & Neff 2009). While a typical study may rely only on 10–15 loci, we identified 118 that have been applied to European wolves (Appendix S4). Out of the 25 institutes that generated genetic data for European wolves, 21 applied microsatellite markers in one or more populations, which has resulted in almost all populations being investigated by at least two institutes (and up to eight; Fig. 2a); the exception is Sierra Morena, for which we found no genetic information. The number of markers applied per population ranged from 17 (in the Carpathians) to up to 76 (in NW Iberia); there was strikingly little overlap between institutes in the markers applied (no marker overlap in five populations for which 76, 41, 30, 25 or 23 markers have been applied; Fig. 2b). The majority of markers have been applied by only one or two institutes (Fig. 3a), and only one-third of the markers have been applied to more than two European populations (Fig. 3b). These striking numbers reflect a clear limitation: comparison of datasets between populations is very challenging, if not impossible. Some overlap exists, reflected in the application of 22 loci in more than half of the populations (in bold in Appendix S4). The extent of this overlap, however, does not follow geographic proximity, which may be used as a proxy for population similarity (Fig. 4), suggesting that the selection of markers used to study a particular population has been based on other, perhaps more practical, reasons (e.g. availability of protocols, markers, or connected researchers).

SNPS

The statistical power and genotyping resolution that can be achieved with a few highly polymorphic microsatellite loci made them the marker of choice for the majority of population genetic studies on wolves to date. In the last few years, however, panels of SNPs have become increasingly popular (vonHoldt et al. 2011, Stronen et al. 2013, Monzón et al. 2014, Pilot et al. 2014). SNPs are directly comparable between laboratories and, therefore, they can be readily incorporated in shared databases. Because of the binary nature of most of them, a much larger set of loci is required to gain the same statistical power as with microsatellites (Hoban et al. 2014). Yet, new multiplex approaches, such as chip-based arrays, allow high-throughput analysis. To date, various SNP arrays have been applied to European wolf populations (Appendix S5). Stronen et al. (2013) and Pilot et al. (2014) generated genotyping data for tens of thousands of SNP loci using canine chip arrays (Appendix S5) to study the evolutionary history of the southern and eastern European wolf populations. Extensive sampling of the entire genome is expected to offer greater accuracy when estimating, for instance, inbreeding, past population bottlenecks and diversifying selection (Pilot et al. 2014).

The implementation of these techniques in applied wildlife monitoring has, however, been hampered until now by their reliance on high quantity and quality of DNA templates; this limits their applicability to non-invasively collected samples (Seddon et al. 2005, Morin & McCarthy 2007). Current genomic knowledge has allowed the design of assays that permit allele-specific amplification of small amplicons (ca. 100 bp), which, coupled with rapid detection

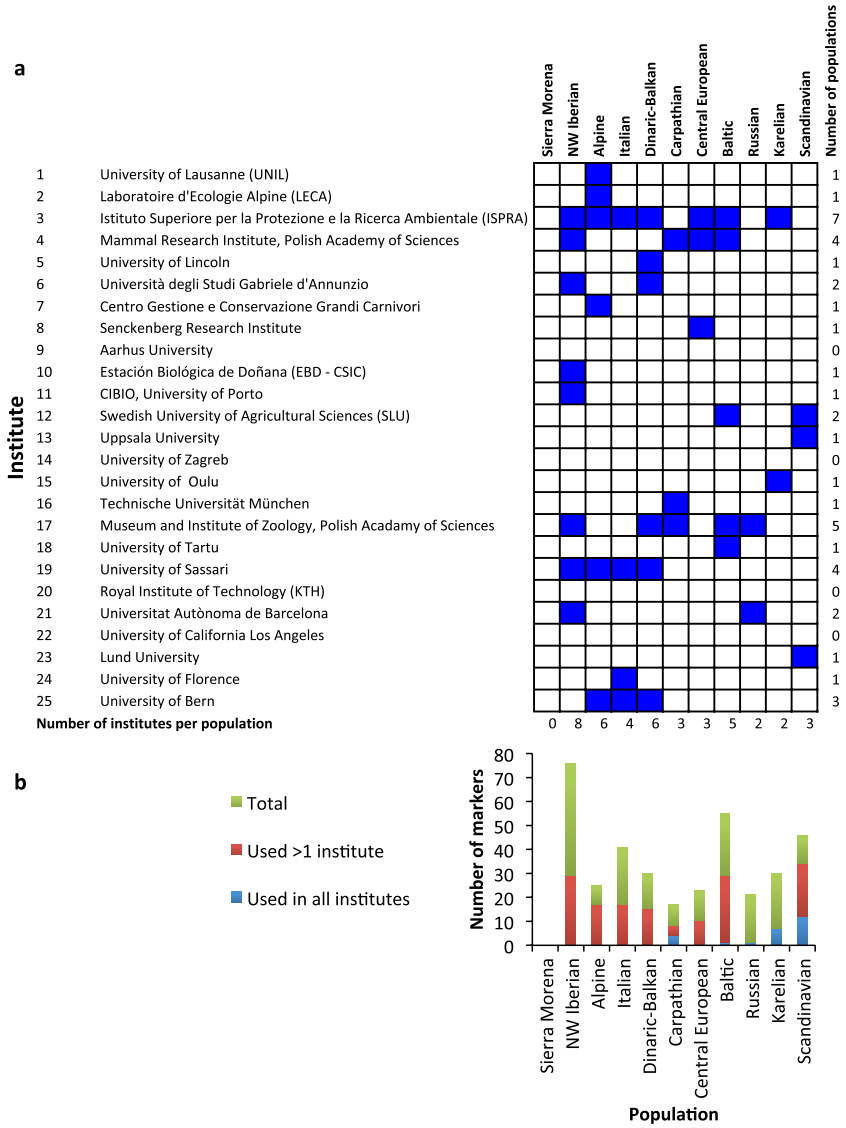

Fig. 2. Institutes listed in Table 2 as hosting microsatellite data, and numbers of markers applied, per wolf population in Europe. (a) For each wolf population, a blue box indicates that the institute collected microsatellite data. (b) Total number of markers applied per population, and overlap in the markers used across institutes. Figure based on the information in Appendix S4.

methods, allow the efficient genotyping of samples with low DNA quality and quantity (e.g. Kraus et al. 2014, see later).

FUNCTIONAL VARIATION

Genetic variants that have detrimental or adaptive effects are of great importance in conservation (Hedrick 2004). In most vertebrates studied to date, balancing selection has been found to operate to maintain diversity in major histocompatibility complex (MHC) genes, and several studies provided evidence that MHC may significantly influence fitness (Bernatchez & Landry 2003). In grey wolves, variation in MHC genes in individuals from Croatia and Finland was compatible with balancing selection (Niskanen et al. 2014), while in the Scandinavian grey wolf population, variation in MHC genes was compatible with expectations under neutrality, probably as a consequence of only three adults contributing to the re-establishment of this population (Seddon & Ellegren 2004). Niskanen et al. (2014) found that grey wolves from Finland that were heterozygote for three MHC class II genes were less often infected by *Trichinella* spp., and carriers of specific MHC alleles, SNP haplotypes, and SNP alleles had fewer helminth infections, in what is a compelling example linking genetic variation with disease resistance. Another example of adaptive variation in grey wolves comes from the presence of a dominant allele in a beta-defensin locus (*CBD103* or *K* locus), which makes the carrier of the allele black (Anderson et al. 2009). Only black heterozygotes experienced higher fitness, probably because of a response mediated by the beta-defensin locus, probably in relation to cellular immunity, and not to the colour itself (Coulson et al. 2011). These few studies in

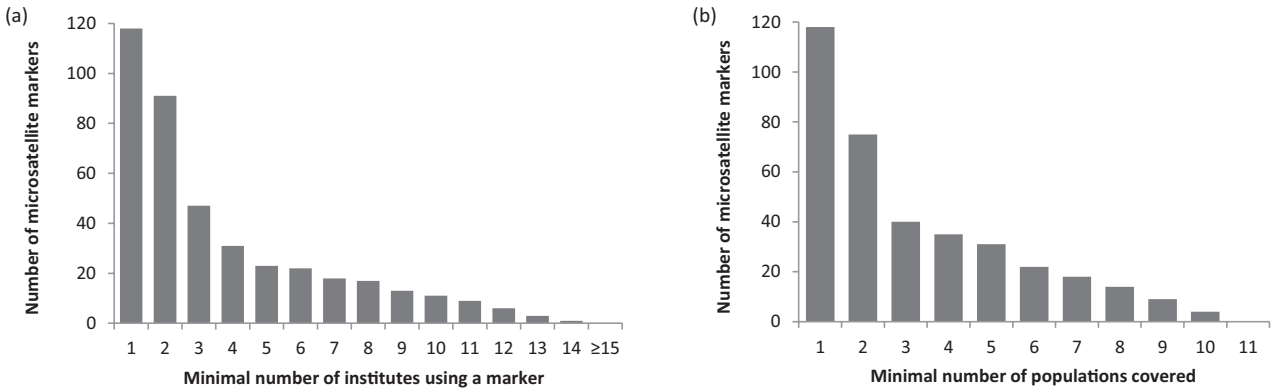

Fig. 3. Number of microsatellite markers used per institute and per population. (a) Number of markers applied by a minimum number of institutes. For example, all 118 markers have been applied by at least one institute, but only 47 of them have been applied by three or more institutes. (b) Number of microsatellite markers that have been applied to a minimum number of populations. Figure based on the information in Appendix S4.

which researchers have attempted to investigate functional variation in grey wolves exemplify that, although adaptive variation holds much promise for the field of conservation genetics, identifying variants with adaptive effects is still elusive.

Current range expansions raise opportunities and needs for further study

Over the past decades, genetic studies on wolves have provided significant improvements in our understanding of the ecology, diversity, evolution, and phylogeographic history of this species in Europe. Studies based on mtDNA sequences have revealed significant differentiation at the local scale (Pilot et al. 2006, 2010), but also the absence of a clear large-scale genetic structure both worldwide and within Europe (Vilà et al. 1999). Two haplotype clades can be recognized in Europe, which locally differ in frequency, but show overlapping distributions (Pilot et al. 2010). Private control region haplotypes may allow for population assignment of individuals of unknown origin, such as in the Italian and Alps populations (Valiere et al. 2003), while others are common in multiple populations, which partly reflects the large dispersal capacities of female wolves.

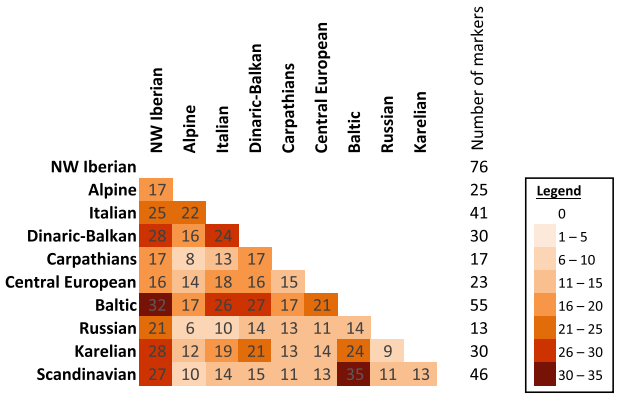

Fig. 4. Pairwise overlap of microsatellite markers between grey wolf populations in Europe. The number of overlapping markers is indicated inside each box. Figure based on the information in Appendix S4.

Microsatellite-based and especially SNP-based studies (e.g. Aspi et al. 2009, Czarnomska et al. 2013, Stronen et al. 2013) have shown a clearer distinction in spatial genetic clusters, allowing the identification of different populations or subpopulations. More research is needed, however, to identify the factors that best explain such structure. Patterns in gene flow may be affected, for instance, by natal-biased dispersal based on learned behaviours related to prey presence, landscape features and habitat differentiation (Pilot et al. 2006, Muñoz-Fuentes et al. 2009, Czarnomska et al. 2013). These studies show genetic differentiation between populations that may occur in the absence of physical barriers to movement and may be explained by ecological factors influencing patterns of dispersal. Allee effects in the edge of an expanding population, because of the decreased probability of finding a mate at low densities, may in part explain the slow spread of a recolonizing population (Hurford et al. 2006) or hybridization with dogs (Muñoz-Fuentes et al. 2010). In addition, patterns of gene flow may change due to demographic factors, as increasing population sizes may result in more regular long-distance dispersal of young wolves. Such dispersals have been documented, for example, from Finland to southern Scandinavia and vice versa (Vilà et al. 2003a), from the northern Apennines in Italy to the western Alps in France (Ciucci et al. 2009) and to eastern Spain (Lampreave et al. 2011), and, more recently, from Slovenia to northern Italy (Ražen et al. 2013).

Recolonization and immigration events shape patterns of genetic variation and the genetic composition of recently established populations. For example, colonization by a limited number of immigrants from a single source resulted in strong founder effects, including genetic differentiation as well as low local diversity, in Scandinavia (Vilà et al. 2003a, Liberg et al. 2005), the Alps (Fabbri et al. 2007), and northern Spain (Lampreave et al. 2011). Based on a combination of mtDNA and microsatellites, Czarnomska et al. (2013) showed that the establishment of a population in western Poland and eastern Germany could mainly be attributed to wolves arriving from north-eastern Poland, although the presence of certain unique alleles suggested an influx from other undersampled areas, probably further to the east or northeast. Sightings and genetic evidence of wolf presence have been documented in western Germany, the Netherlands, Denmark and northern France. Most of these migrants derive from source packs in eastern Germany and western Poland; a few arrivals are from further south (Carpathians, Alps) and from the north-east (north-eastern Poland, Baltic States; Gravendeel et al. 2013, Andersen et al. 2015, Harms & Nowak unpublished data). Future regular immigration and/or continuous wolf presence in central and western Europe is considered likely, and is expected to result in an extension of the currently implemented non-invasive genetic monitoring programmes to understand dispersal routes in the coming years.

Optimizing future research based on available methods and datasets

Wolf genetic studies have provided researchers with: 1) an extensive knowledge of the structure of populations; 2) a large toolkit of methods for answering a wide range of applied and fundamental questions; and 3) considerable numbers of genetic data based on these diverse methods. Future research will benefit from these resources, and should adopt measures to optimise this toolkit whenever possible. This will result in increased efficiency of monitoring programmes for new populations, and will in fact be a necessity for studying large-scale recolonization patterns. If high-resolution data are available for potential source populations, assignment tests for putative migrants can be used to gain evidence of colonization routes. Such assignment tests will depend on the availability of reference collections, based at least largely on the same markers. In practice, we see a number of challenges to achieve this.

DATA ACCESS

Data for wolf European populations are being and/or have been collected by different research groups, and cooperation will be required to gain access to appropriate datasets. This is particularly relevant when attempting to identify the population of origin of a dispersing individual using assignment tests. In such a situation, to determine whether a recent immigrant was sampled earlier elsewhere, one possibility is to send its genotype to different data owners, who then search for a match in their databases. This is, however, a slow process, which may only work on an occasional basis. More generally, researchers or managers may wish to estimate the likelihood of finding a genotype in selected populations, in order to identify the most likely origin of the newly arriving disperser. These two examples illustrate that sharing all genotypes from all populations in a single online database would be highly beneficial for tracing movements and understanding wolf dispersal. Open access to complete datasets will be difficult to accomplish, but various alternatives can be envisioned, such as setting proper restrictions with respect to data ownership, or perhaps an online portal to allow automated queries without full access to the underlying data.

LINKING DATASETS

Our survey of currently applied markers highlights two major challenges: 1) most data for European populations are based on microsatellites, which cannot readily be compared between laboratories; and 2) for microsatellites and mtDNA (and to some extent, also for SNPs), large variation exists in the set of markers implemented. As a result, profiles gathered by different institutes and/or for different populations are not fully compatible. At the moment, this limits the possibility of direct comparison of datasets.

The best strategy to overcome these challenges depends strongly on the marker system applied. MtDNA sequences and microsatellite markers still form the basis of most monitoring programmes. Sequence information can be readily shared via online databases and is relatively easy to obtain for newly available samples. Most sequences collected to date match a 230- or 661-bp fragment of the mtDNA control region (Pilot et al. 2010). It is desirable for newly collected sequences to overlap as much as possible with those sequences already in the online repositories that have been shown to provide high resolution for species and population discrimination. In addition, an agreement on a common nomenclature for haplotypes is desirable, although this may be difficult to achieve as long as sequences do not overlap with each other exactly. An effort has been made already to match newly collected haplotypes (e.g. Leonard et al. 2005, Muñoz-Fuentes et al. 2009, 2010) with previously collected ones (e.g. Vilà et al. 1999), and to make them available through the Genbank online repository. In addition, a supplementary table provided by Pilot et al. (2010) lists matching mtDNA haplotypes from earlier papers, which should facilitate integration of datasets. With

the increasing availability and rapidly decreasing costs of high-throughput sequencing, complete mtDNA genome sequencing is becoming more common. This technique would may overcome the problem of using different fragments and allow researchers to uncover phylogeographic structure and demographic history that was not previously detected with shorter fragments (e.g. Keis et al. 2013).

Regarding microsatellites, adoption of a minimum set of common markers would be ideal. Earlier we reported that a total of 22 loci have been applied in more than half of the populations (in bold in Appendix S4). In fact, four loci (C250, FH2079, FH2088 and FH2096) have been used in all populations, although not in all studies. We recommend that researchers should use these loci whenever possible. Given the long-term monitoring programmes and associated datasets that are already in place, we propose the development of a reference sample collection (either DNA extracts or, preferably, tissue materials) representative of the genetic variation in all European populations, and its exchange among researchers, in an attempt to allow for the comparison of genotypes collected by different laboratories. For the brown bear, a 'yardstick' reference population has been proposed (Skrbinsek et al. 2012) that should ideally contain a large sample size, a large number of genotyped loci and high genetic diversity. Such a reference set can be used to calibrate and enable comparisons of genetic diversity indices across markers obtained in different laboratories. Moreover, the inclusion of the method in the R software package diveRsity makes it easily applicable to other species and studies (Keenan et al. 2013). A similar approach should also be useful for the grey wolf, regardless of the fact that the microsatellite markers collected for different populations overlap to a lesser extent than is the case for the brown bear (Skrbinsek et al. 2012).

Valuable steps along these lines have already been made by several groups studying single or neighbouring wolf populations. The Wolf Alpine Group, a consortium consisting of experts from Italy, France, Switzerland, Germany, Austria and Slovenia, share a common set of markers (Anonymous 2014). The CEwolf consortium (http://www.senckenberg.de/CEwolf), established by researchers from Germany, Poland, Denmark and the Netherlands to study wolves from central Europe, share methodologies and a common set of reference samples. Similar initiatives exist in Scandinavia (SKANDULV), in which Norwegian and Swedish laboratories share markers, and in the Baltic states (BALTWOLF), encompassing Estonia, Latvia and Lithuania. Cooperation via consortia is the only efficient way to achieve Europe-wide integration of microsatellite data for wolves.

In addition, as for mtDNA (earlier), adoption of a common nomenclature is important. A specific effort in this direction has been made by researchers at the genetic laboratories involved in the Wolf Alpine Group, by cloning and sequencing microsatellite alleles obtained from shared reference samples and using the number of repeat units within the microsatellite motifs as a code (Fumagalli 2014). However, this approach is costly and time consuming. New approaches based on next-generation sequencing can be exploited to avoid these limitations and the calibration problems. For instance, tagged microsatellite markers (preferentially short ones, which are more suitable in the case of degraded DNA) can be multiplexed and amplified by polymerase chain reaction and then sequenced using high-throughput sequencing protocols (De Barba et al. 2014). This allows characterization of each allele based on sequence data, instead of the relative length of amplification products separated by electrophoresis, and would allow for direct comparison and exchange of data between laboratories.

Unlike microsatellite genotyping data based on electrophoresis, SNP genotyping data are readily exchangeable between laboratories, as the data are not platform-dependent, and allele assignment can be almost fully automated. The rapidly dropping costs of SNP genotyping and the availability of new techniques, such as the microfluidic dynamic array-based genotyping assay (Fluidigm Corp., San Francisco, CA, USA; Norman et al. 2013, Kraus et al. 2014, Nussberger et al. 2014), potentially allow the analysis of non-invasively collected samples, and may result in increasing application of these markers in the coming years. Newly developed panels are available for both grey wolves (Kraus et al. 2014) and brown bears (Norman et al. 2013). Incorporating SNPs located in the autosomes, as well as in the mtDNA genome and on the Y chromosomes, would allow the development of a single panel of markers to collect information on variation inherited biparentally, maternally, and paternally, respectively. Such SNP panels or next-generation sequencing-based analysis of microsatellites might ultimately replace conventional genotyping of microsatellites, given the advantages these techniques offer in terms of data exchange and speed of sample processing.

The goal and scale of the research project matters when choosing a platform. For example, traditional microsatellite genotyping can be easily up- and downscaled to the number of samples that need to be processed, while SNP genotyping using chips does not offer this flexibility. When processing few samples (e.g. monitoring sporadic individuals or ad hoc genotyping of a low number of samples to support rapid management decisions), microsatellites might be the cheapest option, but only a small increase in sample number may result in reduced costs through SNP genotyping. To give an idea of relative differences in prices, we have estimated costs for these analyses in Germany, where salaries might sit somewhere in between the highest and lowest in Europe for this type of work. With respect to the part of the costs that

differ between the platforms (that is, including only costs of consumables and labour for polymerase chain reaction and genotyping, while excluding sample administration and storage, DNA extraction, acquisition of instruments, laboratory and instrument maintenance, statistical analyses, report writing, database and protocol management), we estimate that processing 24 samples results in similar costs either for microsatellites or SNP genotyping (15 microsatellites, non-invasive samples with four replicates each, €822 of which 42% labour; Fluidigm SNP chip, 96 loci, non-invasive samples, two replicates each, €843 of which 23% labour), while processing 240 samples costs €7710 (38% labour) for microsatellites and €4038 (20% labour) using Fluidigm chips.

Implications and recommendations for conservation and wildlife management

Enhanced conservation efforts in recent years have allowed populations of large carnivores to increase, particularly in Europe and in the conterminous USA, but many populations are still small and persist in highly fragmented and human-dominated landscapes. Increasing our understanding of the ecology of large carnivores and of the best ways in which they can coexist with humans will certainly pose a challenge in the years to come. The fact that large carnivore populations may span national and management borders requires the generation of genetic data to be standardized between countries, to allow for large-scale assessments. Practical conservation issues that managers in most European countries will face include, for example, discovering migration routes and quantifying migration rates, calculating effective population sizes, performing viability and vulnerability analyses, determining the value of different neighbouring populations as donors of genetic material, and writing trans-boundary management plans. Trans-boundary research and conservation plans have proved possible and effective in the USA (e.g. Wydeven et al. 2009), and some attempts have been made in this respect also in Europe (e.g. Blanco 2012, Reinhardt et al. 2013). Continent-wide action plans for large carnivores have existed for over a decade (e.g. Boitani 2000), and following their long-standing recommendations, it is now time to start co-ordinating research.

Several recommendations can be drawn from our review. The re-emergence of large carnivores in Europe is a continent-wide process, and will require continent-wide approaches. Molecular genetics has the potential to provide unprecedented insights, but there is an urgent need to overcome the current fragmentation of research and enable the production of relevant, continent-wide studies that are required for successful conservation and management. We recommend the formation of consortia with the purpose of ensuring direct compatibility of data produced in different laboratories. To make these data useful at the large scale, a smart system for rapid, efficient and cheap exchange of genetic information will prove indispensable. We propose that action is directed towards the establishment of shared SNP panels and towards exploring avenues for next-generation sequencing of a common set of microsatellites, as well as towards developing a shared reference sample collection and a common database to exchange data. Intensive collaboration between institutes will strongly facilitate the establishment of these novel approaches, offering the possibility to share expertise and to set up common experiments and ring-tests.

A common and shared approach will, among other benefits, provide: 1) the most cost-effective use of limited financial resources in biodiversity monitoring and conservation, by avoiding repetitive genetic analysis conducted in different laboratories; 2) an increase in public acceptance of new individual wolf occurrences, by linking (long-distance) dispersers to source populations genetically, avoiding the perception that large carnivore presence is the result of illegal or at least well-hidden reintroduction attempts; 3) facilitation of the coexistence between large carnivores and humans, by aiding the implementation of proper management actions supported by genetic analyses, such as rapid predator identification from livestock kills; and 4) a better understanding of the ecological requirements of large carnivores and the spatial and genetic variation therein. We believe that, if successfully applied, such a common and shared approach will provide the best possible foundation for research on and management of these species at the spatial scale that really matters, and will provide a good example for nature conservation science worldwide.

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REFERENCES

Andersen LW, Harms V, Caniglia R, Czarnomska SD, Fabbri E, Jędrzejewska B et al. (2015) Long-distance dispersal of a wolf, *Canis lupus*, in northwestern Europe. *Mammal Research* 60: 163–168.

Anderson TM, vonHoldt BM, Candille SI, Musiani M, Greco C, Stahler DR et al. (2009) Molecular and evolutionary history of melanism in North American gray wolves. *Science* 323: 1339–1343.

Anonymous (2007) Guidance document on the strict protection of animal species of Community interest under the Habitats Directive 92/43/EEC. European Commission, Brussels, Belgium.

Anonymous (2014) Wolf population status in the Alps: pack distribution and trend up to 2012. http://www.lcie.org

Aspi J, Roininen E, Ruokonen M, Kojola C, Vilà C (2006) Genetic diversity, population structure, effective population size and demographic history of the Finnish wolf population. *Molecular Ecology* 15: 1561–1576.

Aspi J, Roininen E, Kiiskilä J, Ruokonen M, Kojola I, Bljudnik L Danilov P, Heikkinen S, Pulliainen E (2009) Genetic structure of the northwestern Russian wolf populations and gene flow between Russia and Finland. *Conservation Genetics* 10: 815–826.

Bernatchez L, Landry C (2003) MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *Journal of Evolutionary Biology* 16: 363–377.

Blanco JCE (2012) Towards a population level approach for the management of large carnivores in Europe. Challenges and Opportunities, a Large Carinvore Initiative for Europe report prepared for the European Commission (contract N°070307/2012/629085/SER/B3).

Boitani L (2000) *Action Plan for the Conservation of the Wolves (Canis lupus) in Europe*. Council of Europe Publishing, Strasbourg, France.

Bradman H, Grewe P, Appelton B (2011) Direct comparison of mitochondrial markers for the analysis of swordfish population structure. *Fisheries Research* 109: 95–99.

Caniglia R, Fabbri E, Mastrogiuseppe L, Randi E (2013) Who is who? Identification of livestock predators using forensic genetic approaches. *Forensic Science International: Genetics* 7: 397–404.

Caniglia R, Fabbri E, Galaverni M, Milanesi P, Randi E (2014) Noninvasive sampling and genetic variability, pack structure, and dynamics in an expanding wolf population. *Journal of Mammalogy* 95: 41–59.

Chapron G, Kaczensky P, Linnell JDC, von Arx M, Huber D, Andrén H et al. (2014) Recovery of large carnivores in Europe's modern human-dominated landscapes. *Science* 346: 1517–1519.

Ciucci P, Reggioni W, Maiorano L, Boitani L (2009) Long-distance dispersal of a rescued wolf from the northern Apennines to the western Alps. *The Journal of Wildlife Management* 73: 1300–1306.

Coulson T, MacNulty DR, Stahler DR, vonHoldt B, Wayne RK, Smith DW (2011) Modeling effects of environmental change on wolf population dynamics, trait evolution, and life history. *Science* 334: 1275–1278.

Czarnomska S, Jędrzejewska B, Borowik T, Niedziałkowska M, Stronen A, Nowak S et al. (2013) Concordant mitochondrial and microsatellite DNA structuring between Polish lowland and Carpathian Mountain wolves. *Conservation Genetics* 14: 573–588.

De Barba M, Miquel C, Lobreaux S, Boyer F, Swenson JE, Taberlet P (2014) New perspectives for standardized bear genotyping using high throughput sequencing. In: Proceedings of the 23rd International Conference on Bear Research and Management, Thessaloniki, Greece.

Ellegren H, Savolainen P, Rosen B (1996) The genetical history of an isolated population of the endangered grey wolf *Canis lupus*: a study of nuclear and mitochondrial polymorphisms. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 351: 1661–1669.

Fabbri E, Miquel C, Lucchini V, Santini A, Caniglia R, Duchamp C et al. (2007) From the Apennines to the Alps: colonization genetics of the naturally expanding Italian wolf (*Canis lupus*) population. *Molecular Ecology* 16: 1661–1671.

Fabbri E, Caniglia R, Kusak J, Galov A, Gomerčić T, Arbanasić H, Huber D, Randi E (2014) Genetic structure of expanding wolf (*Canis lupus*) populations in Italy and Croatia, and the early steps of the recolonization of the Eastern Alps. *Mammalian Biology – Zeitschrift für Säugetierkunde* 79: 138–148.

Flagstad Ø, Walker CW, Vilà C, Sundqvist AK, Fernholm B, Hufthammer AK, Wiig Ø, Koyola I, Ellegren H (2003) Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. *Molecular Ecology* 12: 869–880.

Fumagalli L (2014) *Standardisation of the Genetic Analyses (Microsatellite Genotyping) Among the Different Laboratories Involved in the Wolf Alpine Project*. Laboratory for Conservation Biology, Department of Ecology and Evolution. University of Lausanne, Switzerland.

Godinho R, Llaneza L, Blanco JC, Lopes S, Álvares F, García EJ, Palacios V, Cortés Y, Talegón J, Ferrand N (2011) Genetic evidence for multiple events of hybridization between wolves and domestic dogs in the Iberian Peninsula. *Molecular Ecology* 20: 5154–5166.

Godinho R, López-Bao JV, Castro D, Llaneza L, Lopes S, Silva P, Ferrand N (2015) Real-time assessment of hybridization between wolves and dogs: combining noninvasive samples with ancestry informative markers. *Molecular Ecology Resources* 15: 317–328.

Gravendeel B, de Groot GA, Kik M, Beentjes KK, Bergman H, Caniglia R et al. (2013) The first wolf found in the Netherlands in 150 years was the victim of a wildlife crime. *Lutra* 56: 93–109.

Gula R, Hausknecht R, Kuehn R (2009) Evidence of wolf dispersal in anthropogenic habitats of the Polish Carpathian Mountains. *Biodiversity and Conservation* 18: 2173–2184.

Harms V, Nowak C, Carl S, Muñoz-Fuentes V (2015) Experimental evaluation of genetic predator identification from saliva traces on wildlife kills. *Journal of Mammology* 96: 138–143.

Hedrick PW (2004) Recent developments in conservation genetics. *Forest Ecology and Management* 197: 3–19.

Hindrikson M, Männil P, Ozolins J, Krzywinski A, Saarma U (2012) Bucking the trend in wolf-dog hybridization: first evidence from Europe of hybridization between female dogs and male wolves. *PLoS ONE* 7: e46465.

Hindrikson M, Remm J, Männil P, Ozolins J, Tammeleht E, Saarma U (2013) Spatial genetic analyses reveal cryptic population structure and migration patterns in a continuously harvested grey wolf (*Canis lupus*) population in north-eastern Europe. *PLoS ONE* 8: e75765.

Hoban S, Arntzen JA, Bruford MW, Godoy JA, Rus Hoelzel A, Segelbacher G, Vilà C, Bertorelle G (2014) Comparative evaluation of potential indicators and temporal sampling protocols for monitoring genetic erosion. *Evolutionary Applications* 7: 984–998.

Hurford A, Hebblewhite M, Lewis MA (2006) A spatially explicit model for an Allee effect: why wolves recolonize so slowly in Greater Yellowstone. *Theoretical Population Biology* 70: 244–254.

Iacolina L, Scandura M, Gazzola A, Cappai N, Capitani C, Mattioli L, Vercillo F, Apollonio M (2010) Y-chromosome microsatellite variation in Italian wolves: a contribution to the study of wolf–dog hybridization patterns. *Mammalian Biology – Zeitschrift für Säugetierkunde* 75: 341–347.

Jansson E, Harmoinen J, Ruokonen M, Aspi J (2014) Living on the edge: reconstructing the genetic history of the Finnish wolf population. *BMC Evolutionary Biology* 14: 64.

Jędrzejewksi W, Branicki W, Veit C, Medugorac I, Pilot M, Bunevich AN et al. (2005) Genetic diversity and relatedness within packs in an intensely hunted population of wolves *Canis lupus*. *Acta Theriologica* 50: 3–22.

Keenan K, McGinnity P, Cross TF, Crozier WW, Prodöhl PA (2013) diveRsity: an R package for the estimation and exploration of population genetics parameters and their associated errors. *Methods in Ecology and Evolution* 4: 782–788.

Keis M, Remm J, Ho SYW, Davison J, Tammeleht E, Tumanov IL et al. (2013) Complete mitochondrial genomes and a noval spatial genetic method reveal cryptic phylogeographical structure and migration patterns among brown bears in north-western Eurasia. *Journal of Biogeography* 40: 915–927.

Kraus RHS, vonHoldt B, Cocchiararo B, Harms V, Bayerl H, Kühn R, Förster DW, Fickel J, Roos C, Nowak C (2014) A single-nucleotide polymorphism-based approach for rapid and cost-effective genetic wolf monitoring in Europe based on noninvasively collected samples. *Molecular Ecology Resources* 15: 295–305.

Lampreave G, Ruiz-Olmo J, Garcia-Petit J, López-Martín JM, Bataille A, Francino O, Sastre N, Ramírez Ó (2011) El lobo vuelve a Cataluña: historia del regreso y medidas de conservación. *Quercus* 302: 16–25.

Leonard JA, Vilà C, Wayne RK (2005) Legacy lost: genetic variability and population size of extirpated US gray wolves (*Canis lupus*). *Molecular Ecology* 14: 9–17.

Liberg O, Andrén H, Pedersen H-C, Sand H, Sejberg D, Wabakken P, Åkesson M, Bensch S (2005) Severe inbreeding depression in a wild wolf (*Canis lupus*) population. *Biology Letters* 1: 17–20.

Liberg O, Chapron G, Wabakken P, Pedersen HC, Hobbs NT, Sand H (2011) Shoot, shovel and shut up: cryptic poaching slows restoration of a large carnivore in Europe. *Proceedings of the Royal Society of London B: Biological Sciences* 279: 910–915.

Linnell JDC, Brøseth H, Solberg EJ, Bainerd SM (2005) The origins of the southern Scandinavian wolf *Canis lupus* population: potential for natural immigration in relation to dispersal distances, geography and Baltic ice. *Wildlife Biology* 11: 383–391.

López-Bao JV, Blanco JC, Rodríguez A, Godinho R, Sazatornil V, Alvares F et al. (2015) Toothless wildlife protection laws. *Biodiversity and Conservation* 24: 2105–2108.

Lucchini V, Fabbri E, Marucco F, Ricci S, Boitani L, Randi E (2002) Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. *Molecular Ecology* 11: 857–868.

Lucchini V, Galov A, Randi E (2004) Evidence of genetic distinction and long-term population decline in wolves (*Canis lupus*) in the Italian Apennines. *Molecular Ecology* 13: 523–536.

Marucco F, Pletscher DH, Boitani L, Schwartz MK, Pilgrim KL, Lebreton J-D (2009) Wolf survival and population trend using non-invasive capture–recapture techniques in the Western Alps. *Journal of Applied Ecology* 46: 1003–1010.

Miller CR, Joyce P, Waits LP (2002) Assessing allelic dropout and genotype reliability using maximum likelihood. *Genetics* 160: 357–366.

Miquel C, Bellemain E, Poillot C, Bessière J, Durand A, Taberlet P (2006) Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. *Molecular Ecology Notes* 6: 985–988.

Monzón J, Kays R, Dykhuizen DE (2014) Assessment of coyote–wolf–dog admixture using ancestry-informative diagnostic SNPs. *Molecular Ecology* 23: 182–197.

Morin PA, McCarthy M (2007) Highly accurate SNP genotyping from historical and low-quality samples. *Molecular Ecology Notes* 7: 937–946.

Muñoz-Fuentes V, Darimont CT, Wayne RK, Paquet PC, Leonard JA (2009) Ecological factors drive differentiation in wolves from British Columbia. *Journal of Biogeography* 36: 1516–1531.

Muñoz-Fuentes V, Darimont C, Paquet P, Leonard J (2010) The genetic legacy of extirpation and re-colonization in Vancouver Island wolves. *Conservation Genetics* 11: 547–556.

Niskanen AK, Kennedy LJ, Ruokonen M, Kojola I, Lohi H, Isomursu M, Jansson E, Pyhäjärvi T, Aspi J (2014) Balancing selection and heterozygote advantage in major histocompatibility complex loci of the bottlenecked Finnish wolf population. *Molecular Ecology* 23: 875–889.

Norman AJ, Street NR, Spong G (2013) *De Novo* SNP discovery in the Scandinavian brown bear (*Ursus arctos*). *PLoS ONE* 8: e81012.

Nussberger B, Wandeler P, Camenisch G (2014) A SNP chip to detect introgression in wildcats allows accurate genotyping of single hairs. *European Journal of Wildlife Research* 60: 405–410.

Pilot M, Jedrzejewski W, Branicki W, Sidorovich VE, Jedrzejewska B, Stachura K, Funk SM (2006) Ecological factors influence population genetic structure of European grey wolves. *Molecular Ecology* 15: 4533–4553.

Pilot M, Branicki W, Jedrzejewski W, Goszczynski J, Jedrzejewska B, Dykyy I, Shkvyrya M, Tsingarska E (2010) Phylogeographic history of grey wolves in Europe. *BMC Evolutionary Biology* 10: 104.

Pilot M, Greco C, vonHoldt BM, Jedrzejewska B, Randi E, Jedrzejewski W, Sidorovich VE, Ostrander EA, Wayne RK (2014) Genome-wide signatures of population bottlenecks and diversifying selection in European wolves. *Heredity* 112: 428–442.

Randi E (2011) Genetics and conservation of wolves *Canis lupus* in Europe. *Mammal Review* 41: 99–111.

Randi E, Lucchini V (2002) Detecting rare introgression of domestic dog genes into wild wolf (*Canis lupus*) populations by Bayesian admixture analyses of microsatellite variation. *Conservation Genetics* 3: 31–45.

Randi E, Hulva P, Fabbri E, Galaverni M, Galov A, Kusak J, Bigi D, Bolfíková BC, Smetanová M, Caniglia R (2014) Multilocus detection of wolf x dog hybridization in Italy, and guidelines for marker selection. *PLoS ONE* 9: e86409.

Ražen N, Castagna C, Kljun F, Ivan K, Miha K, Roman L, Hubert P (2013) Documented long-distance dispersal of wolf (*Canis lupus*) from Dinaric population with successful pack formation. In Proceedings of the V: International Conference Wolf Conservation in Human Dominated Landscapes. University of Ljubljana, Ljubjana, Slovenia.

Reinhardt I, Kluth G, Nowak S, Myslajek RW (2013) A review of wolf management in Poland and Germany with recommendations for future transboundary collaboration. *BfN-Skripten* 356.

Ripple WJ, Estes JA, Beschta RL, Wilmers CC, Ritchie EG, Hebblewhite M et al. (2014) Status and ecological effects of the world's largest carnivores. *Science* 343: 6167.

Savolainen P, Lundeberg J (1999) Forensic evidence based on mtDNA from dog and wolf hairs. *Journal of Forensic Sciences* 44: 77–81.

Seddon JM, Ellegren H (2004) A temporal analysis shows major histocompatibility complex loci in the Scandinavian wolf population are consistent with neutral evolution. *Proceedings of the Royal Society B-Biological Sciences* 271: 2283–2291.

Seddon JM, Parker HG, Ostrander EA, Ellegren H (2005) SNPs in ecological and conservation studies: a test in the Scandinavian wolf population. *Molecular Ecology* 14: 503–511.

Shaw CN, Wilson PJ, White BN (2003) A reliable molecular method of gender determination for mammals. *Journal of Mammalogy* 84: 123–128.

Skrbinsek T, Jelencic M, Waits LP, Potocnik H, Kos I, Trontelj P (2012) Using a reference population yardstick to calibrate and compare genetic diversity reported in different studies: an example from the brown bear. *Heredity* 109: 299–305.

Stronen AV, Jędrzejewska B, Pertoldi C, Demontis D, Randi E, Niedziałkowska M et al. (2013) North-south differentiation and a region of high diversity in European wolves (*Canis lupus*). *PLoS ONE* 8: e76454.

Sundqvist A-K, Ellegren H, Vilà C (2008) Wolf or dog? Genetic identification of predators from saliva collected around bite wounds on prey. *Conservation Genetics* 9: 1275–1279.

Sundqvist AK, Ellegren H, Olivier M, Vilà C (2001) Y chromosome haplotyping in Scandinavian wolves (*Canis lupus*) based on microsatellite markers. *Molecular Ecology* 10: 1959–1966.

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* 24: 3189–3194.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution* 14: 323–327.

Thalmann O, Shapiro B, Cui P, Schuenemann VJ, Sawyer SK, Greenfield DL et al. (2013) Complete mitochondrial genomes of ancient canids suggest a European origin of domestic dogs. *Science* 342: 871–874.

vonHoldt BM, Stahler DR, Smith DW, Earl DA, Pollinger JP, Wayne RK (2008) The genealogy and genetic viability of reintroduced Yellowstone grey wolves. *Molecular Ecology* 17: 252–274.

vonHoldt BM, Pollinger JP, Earl DA, Knowles JC, Boyko AR, Parker H et al. (2011) A genome-wide perspective on the evolutionary history of enigmatic wolf-like canids. *Genome Research* 21: 1294–1305.

Valiere N, Fumagalli L, Gielly L, Miquel C, Lequette B, Poulle ML, Weber J-M, Artlettaz R, Taberlet P (2003) Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years. *Animal Conservation* 6: 83–92.

Vilà C, Amorim IR, Leonard JA, Posada D, Castroviejo J, Petrucci-Fonseca F et al. (1999) Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. *Molecular Ecology* 8: 2089–2103.

Vilà C, Sundqvist A-K, Flagstad Ø, Seddon J, Björnerfeldt S, Kojola I, Casulli A, Sand H, Wabakken P, Ellegren H (2003a) Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant. *Proceedings of the Royal Society of London, series B* 270: 91–97.

Vilà C, Walker C, Sundqvist AK, Fagstad Ø, Andersone Z, Casulli A, Kojola I, Valdmann H, Halverson J, Ellegren H

(2003b) Combined use of maternal, paternal and bi-parental genetic markers for the identification of wolf-dog hybrids. *Heredity* 90: 17–24.

Wabakken P, Sand H, Kojola I, Zimmermann B, Arnemo JM, Pedersen HC, Liberg O (2007) Multistage, long-range natal dispersal by a global positioning system-collared Scandinavian wolf. *The Journal of Wildlife Management* 71: 1631–1634.

Wayne RK, Lehman N, Allard NW, Honeycutt RL (1992) Mitochondrial DNA variability of the grey wolf: genetic consequences of population decline and habitat fragmentation. *Conservation Biology* 6: 559–569.

Wong AK, Neff MW (2009) DOGSET: pre-designed primer sets for fine-scale mapping and DNA sequence interrogation in the dog. *Animal Genetics* 40: 569–571.

Wydeven AP, Jurewicz RL, Van Deelen TR, Erb J, Hammill JH, Beyer DE Jr, Roell B, Wiedenhoeft JE, Weitz DA (2009) Gray wolf conservation in the Great Lakes region of the United States. In: Musiani M, Boitani L, Paquet PC (eds) *A New Era for Wolves and People: Wolf Recovery, Human Attitudes, and Policy*, 69–85. University of Calgary Press, Calgary, Canada.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Appendix S1. List of publications dealing with genetic studies of grey wolves in Europe, used as the basis for a methodological review

Appendix S2. Pairs of primers used to amplify and sequence mitochondrial DNA in grey wolves in Europe, and populations for which data have been gathered.

Appendix S3. Markers located on the Y chromosome studied in grey wolves in Europe and populations for which data have been gathered.

Appendix S4. Microsatellite loci used to study grey wolves in Europe, and list of populations for which data have been gathered.

Appendix S5. Available SNP panels and their application on grey wolves in Europe.

Appendix S6. References cited in Appendices S2–S5.