

# Long-distance dispersal of a wolf, *Canis lupus*, in northwestern Europe

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**Abstract** Several mammal species have recolonized their historical ranges across Europe during the last decades. In November 2012, a wolf-looking canid was found dead in Thy National Park (56° 56' N, 8° 25' E) in Jutland, Denmark. DNA from this individual and nine German wolves were genotyped using a genome-wide panel of 22,163 canine single nucleotide polymorphism (SNP) markers and compared to existing pro-

files based on the same marker panel obtained from northeastern Polish ( $n=13$ ) wolves, domestic dogs ( $n=13$ ) and known wolf-dog hybrids ( $n=4$ ). The Thy canid was confirmed to be a wolf from the German-western Polish population, approximately 800 km to the southeast. Access to the German reference database on DNA profiles based on 13 autosomal microsatellites of German wolves made it possible to pinpoint the exact pack origin of the Thy wolf in Saxony, Germany. This was the first documented observation of a wolf in Denmark in 200 years and another example of long-distance dispersal of a carnivore.

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## Introduction

During the last few decades, populations of several mammal species that had declined in the past from various anthropogenic pressures (Rosser and Mainka 2002) started a gradual recolonization of their historical ranges across Europe (Chapron et al. 2014). Wolves (*Canis lupus*), for instance, were historically distributed throughout Europe, but many populations declined toward extinction in the eighteenth and nineteenth centuries, mainly due to persecution and overharvesting (Randi 2011 and references therein). Isolated populations survived in Iberia and southern Italy, while larger wolf populations remained in eastern Europe (Linnell et al. 2008; Kaczensky et al. 2013).

Strict legal protection of wolves, together with the recovery of natural prey populations, are probably the main causes for the recent recovery and range expansion of European wolf populations (Chapron et al. 2014; Nowak et al. 2011; Randi

2011). Wolves from Poland recolonized Germany in the late 1990s and are now expanding their range from the first established core area in the Lausitz region (near the Polish border, Fig. 1) toward Lower Saxony and Schleswig-Holstein in the north-west (Reinhardt et al. 2013). Wolves dispersing from the Lausitz region have also been recorded in other countries such as Belarus (Reinhardt and Kluth 2011). In November 2012, the carcass of a wolf-like canid was discovered in Thy National Park (56° 58' N, 8° 26' E) in Jutland, Denmark, where wolves have been absent since 1813 (Jensen 1993). Morphological examination revealed a male canid that had died from natural causes. The objective of our analysis was to identify 1) proper species and 2) geographical origin of this canid. To address these questions, we chose a genome-wide approach with data from a panel of dog, *Canis lupus familiaris*, single nucleotide polymorphism (SNP) markers. To determine the geographical origin, Germany and northeastern Poland were chosen as possible sources based on the results of the study by Czaromska et al. (2013) who found that wolves colonizing western Poland and eastern Germany were genetically similar and likely derived from wolves in northeastern Poland with ongoing gene flow likely connecting the regions. Subsequently, clustering analyses with wolf samples from northeastern Poland and Germany, domestic dogs and known wolf-dog hybrids were performed based on SNP markers, and furthermore, we performed

traditional microsatellite analysis, followed by comparison with a wolf genotype database searching for an individual DNA profile match.

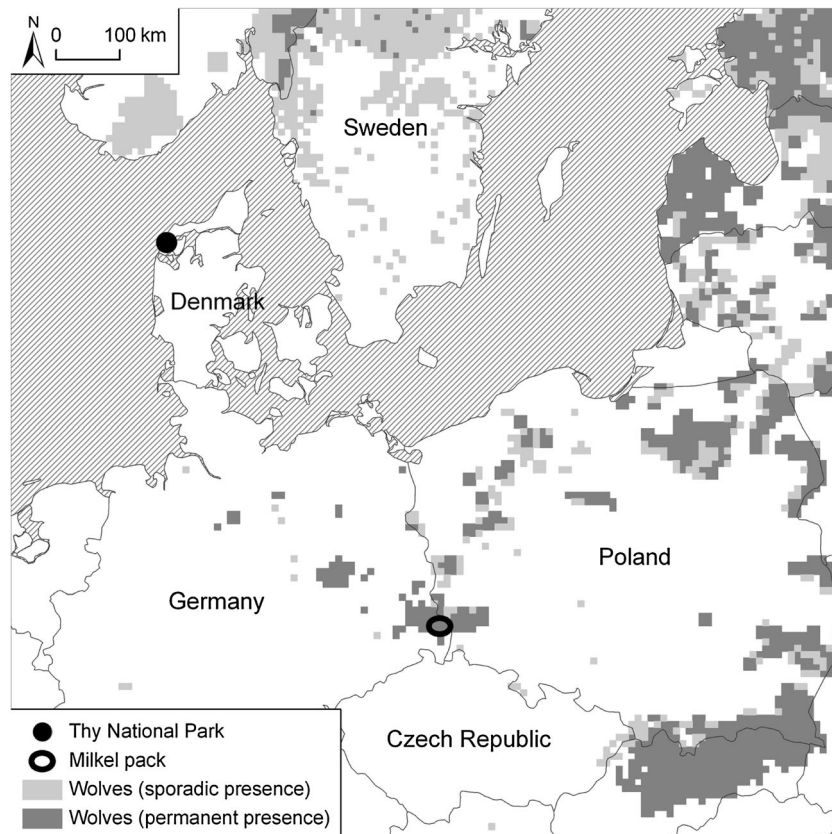
## Materials and methods

### SNP genotyping

We compared DNA from a tissue sample of the Thy canid with the genetic profiles of known wolves, domestic dogs and hybrids. Reference profiles included wolves from Germany ( $n=9$ , analysed in the present study) and mainly northeastern Poland (a few originated from western Poland) ( $n=13$ ), domestic dogs ( $n=13$ , of Italian origin) and known (captive) male wolf  $\times$  female dog first-generation (F1) hybrids ( $n=3$  of Italian origin) and one German hybrid (analysed in the present study). The Italian and Polish profiles were available from earlier research (Stronen et al. 2013). Polish samples were selected from known source areas for westward recolonization (western and northeastern Poland) (Czaromska et al. 2013). German samples were obtained from the genetic reference database for German wolf monitoring at Senckenberg Research Institute, Germany.

Genotyping was performed with the Illumina® CanineHD BeadChip microarray with 170,000 SNP loci (Illumina, Inc.

**Fig. 1** Map indicating permanent and sporadic presence of wolves in northwestern Europe (Kaczensky et al. 2013). Black circle the Milkel pack in the Lausitz region, eastern Germany; black dot Thy National Park, Denmark



San Diego, CA, USA) at AROS Applied Biotechnology A/S in Aarhus, Denmark. Preliminary results were screened for genotyping quality as described in Stronen et al. (2013). PLINK (Purcell et al. 2007) was used to merge the Thy and German data with northeastern Polish and Italian reference samples based on 27,353 SNPs pruned for linkage disequilibrium (Stronen et al. 2013). The resulting dataset was screened in PLINK with the following parameters: minor allele frequency >0.01 and maximum per SNP missing rate <0.02 (Stronen et al. 2013). Observed and expected heterozygosity and percentage of missing and polymorphic loci for wolf population clusters were estimated with PLINK and R 2.15.1 (R Development Core Team 2008). Population structure was examined by principal component analyses (PCA) performed with the *Adegenet* package (Jombart 2008) in R ([www.r-project.org](http://www.r-project.org); R Development Core Team 2008).

#### Microsatellite analysis

DNA extracted from the Thy canid tissue sample was amplified with 13 unlinked autosomal microsatellite loci commonly used in wolf genetics studies: CPH5 (Fredholm and Winterø 1995); FH2001, FH2010, FH2017, FH2054, FH2087, FH2088, FH2097, FH2137, FH2040 and FH2161 (Francisco et al. 1996); vWF (Shibuya et al. 1994); and PEZ17 (Neff et al. 1999).

Three multiplex PCRs were performed in 10- $\mu$ l reactions containing HotStarTaq Master Mix (Qiagen), 0.2  $\mu$ M of each primer, 2 ng BSA and genomic DNA. PCRs were run in a T1 plus Thermocycler (Biometra GmbH) with an initial denaturation step of 95 °C for 3 min followed by 5 cycles of 94 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s; another 5 cycles of 94 °C for 30 s, 58 °C for 90 s and 72 °C for 60 s; 5 cycles of 94 °C for 30 s, 54 °C for 90 s and 72 °C for 60 s; 20 cycles of 94 °C for 30 s, 54 °C for 90 s and 72 °C for 60 s; and a final extension of 72 °C for 30 min. PCR products were electrophoresed on a ABI3730 DNA Analyzer (Life Technologies). The resulting multilocus genotype was compared with the genetic reference database for German wolf monitoring at Senckenberg Research Institute, Germany.

#### Results

We obtained individual profiles based on 22,163 SNPs, which were used in further analyses. For the Thy and German individuals, at least 95 % of the shared SNPs were called for each canid. The percentage of missing loci was higher in northeastern Polish (0.24) than in the German (0.06) individuals. The observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosity and percentage of polymorphic loci ( $P$ ) were higher in individuals sampled mainly in northeastern Poland ( $H_O=0.2644$  (SE 0.001),  $H_E=0.2576$  (SE 0.001),  $P=82.6$ ) than in Germany ( $H_O=$

0.2167 (SE 0.001),  $H_E=0.1827$  (SE 0.001),  $P=55.4$ ). For northeastern Polish and German wolves, none of the loci deviated significantly from Hardy-Weinberg expectations after an overall Bonferroni correction (Rice 1989) for multiple comparisons ( $P$  threshold=0.05) (data not shown).

The Thy canid clearly clustered with wolves in the PCA and specifically among individuals sampled in Germany (Fig. 2). The northeastern Polish wolves differed clearly from the German wolves analysed. In contrast, as expected, the known hybrids clustered in positions intermediate between wolves and domestic dogs. Furthermore, one individual sampled in Germany clustered with the wolves sampled in northeastern Poland.

Comparison of the microsatellite multilocus genotype revealed a perfect match of the Thy canid with one of the known individuals from the German wolf genotype database, a male identified in Saxony. Earlier genetic analysis (data not shown) has identified this individual as progeny of the Milkel pack in the Lausitz region, the core area of the wolf distribution in Germany.

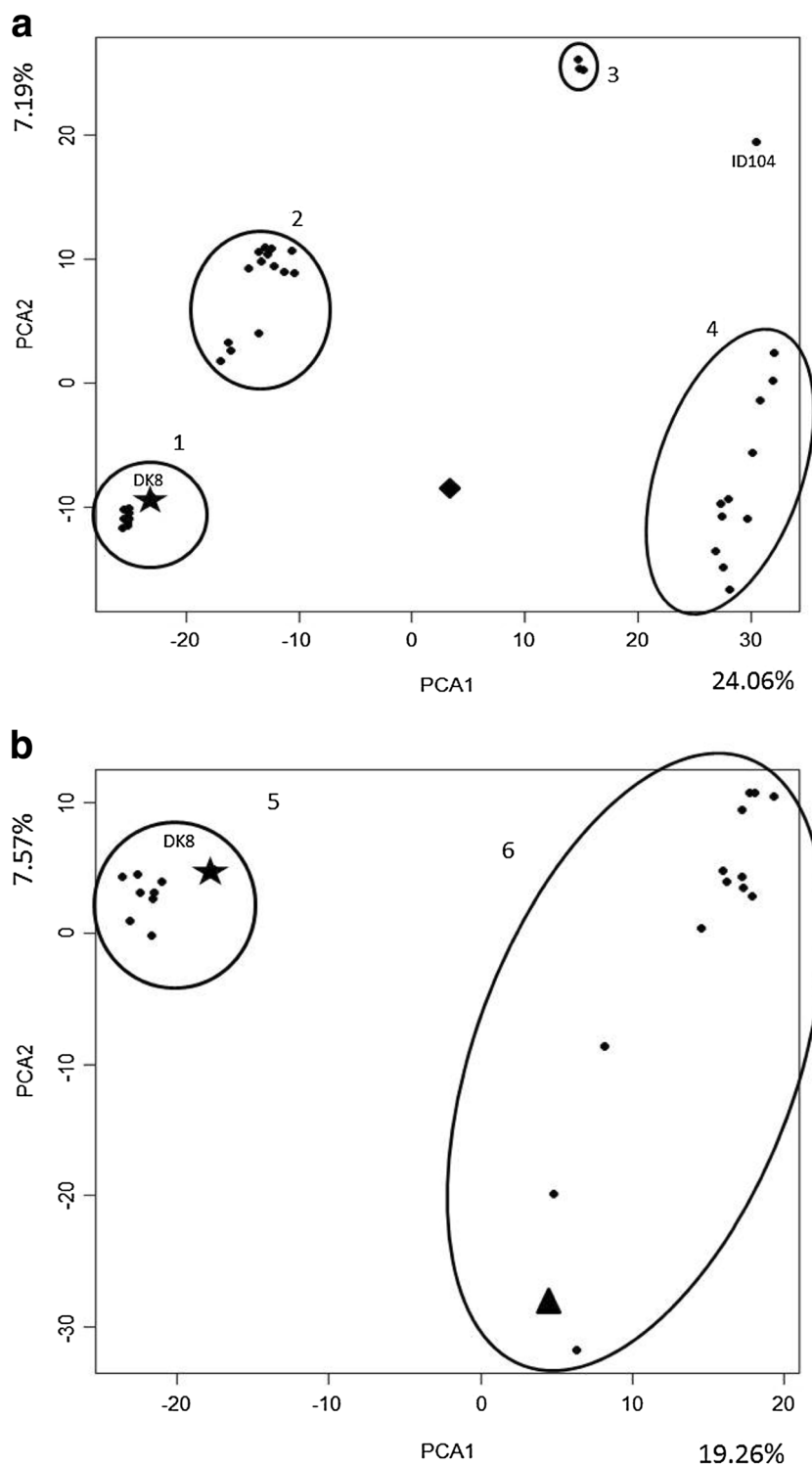
#### Discussion

Our SNP results unambiguously identified the Thy canid as a wild wolf, which likely originated from the German-western Polish (Lausitz—Lower Silesia) population and represents the first wolf reported in Denmark for over 200 years. Both northeastern Polish and German wolves showed higher observed than expected heterozygosity, but the discrepancy was larger in German wolves and seemed consistent with a founder effect. However, one German sample appeared to have a more northeastern Polish origin, which suggests a recent immigration event.

There was no indication of domestic dog ancestry in the Thy wolf nor in other wolves from Germany. However, the known past occurrence of a German hybrid and different reports from northern Europe (e.g. Hindrikson et al. 2012; Stronen et al. 2013), Italy (Caniglia et al. 2014; Randi et al. 2014), Spain (Godinho et al. 2011) and North America (Monzón et al. 2014) suggest continuous monitoring for hybrids is required. Wolves and dogs show clear differentiation with genome-wide SNP profiles (e.g. vonHoldt et al. 2011), and the use of Italian dog reference samples is unlikely to have limited the ability to identify hybrids.

Modern genomic methods represent powerful new tools for the identification and monitoring of wild organisms. However, the interpretation of subsequent high-resolution data should be done with caution. It is important to determine whether genetic clusters represent family groups (i.e. wolf packs) or higher levels of population structure such as separate subpopulations or populations. The present case suggested that the German wolves were genetically different from the northeastern Polish

**Fig. 2** **a** Principal component analysis (PCA) of the German and northeastern Polish wolves, domestic dogs and hybrids based on 22,163 SNPs showing the clustering of the Danish individual. *Star* DK8. **1** German wolves, **2** northeastern Poland wolves, **3** hybrids and **4** domestic dogs. ID104 (outlier Italian dog) might be wolf-dog hybrid  $\times$  dog backcross. *Diamond* German wolf-dog hybrid. **b** PCA without the domestic dogs and hybrids; **5** German wolves, **6** northeastern Polish wolves and *triangle* German wolf



wolves; however, whether this was attributed to family/founder events or actually subpopulations is inconclusive due to the limited sample size. Our ability to confirm the Thy canid as a (non-hybridized) wild wolf from the German-western Polish population was facilitated by the availability of high-quality tissue after the individual was found dead. However, new noninvasive genomic methods

(e.g. Kraus et al. 2014) represent important contributions toward elucidating wildlife movements (Deinet et al. 2013).

The genetic profile based on 13 microsatellite markers combined with field observations showed that the Thy wolf had first-order relatives (father and brother) in the Milkel pack from the Lausitz region (data not shown). This result pinpointed the origin of the Thy wolf and corroborated the



result of the SNP analysis, which was performed prior to access to the German reference database. Moreover, the observed microsatellite DNA profile was located in Northern Germany mid-2012, a couple of months before the wolf was found dead in Thy, Denmark which suggested that the Thy wolf reached Denmark by active dispersal (Harms, personal communication).

A recent review (Chapron et al. 2014) on the recovery of large carnivores in Europe considers the present coexistence of humans and large carnivores today a result of the joined conservation effort of the countries during the past three decades. The wolf has recovered to around 12,000 individuals in Europe. This is, in part, a result of the species' ability to colonize a diverse range of anthropogenically modified habitats, reflecting its high grade of adaptability.

The wolf's long-distance dispersal propensities call for some anticipatory research such as recognizing and modelling suitable habitats in Denmark as performed by Fechter and Storch (2014) for the wolves in Germany and by Jędrzejewski et al. (2008) for western Poland. The latter study found that wolves may settle fast in the areas predicted as good potential habitats for this large carnivore. Landscape permeability analyses identifying possible factors that may determine migration routes for long-distance dispersal (e.g. Huck et al. 2011) would also be of high importance, especially in western European countries with dense transportation infrastructure.

The number of wolves and their ecological role as predators are at present limited in Denmark and Germany. Importantly, however, the wolf is a native species that historically was part of the ecosystem and appears to play a key role in neighbouring countries where its ecological function has persisted over time (Kuijper et al. 2013). Whereas it is well established that wolves in northern Europe occasionally travel >1000 km (Wabakken et al. 2007), this first observation of wolves in Denmark since 1813 (Jensen 1993) represents an exciting example of native wildlife recolonizing historic ranges from which they have been absent for centuries. Identical trends of an ongoing rapid dispersal of wolves (Giucci et al. 2009; Duchamp et al. 2012) are observed in other western and central European countries including France and Germany.

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