UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE

Wolf in Denmark – Genetic Species Identification

Christina Næsborg-Nielsen (gft749)
Lene Bruhn Pedersen (tdw543)

Supervisor: Anders Johannes Hansen
Lab supervisor: Alba de la Iglesia
Geogenetics
University of Copenhagen
16th of June 2016

Table of contents

Abstract:

Ulven (*Canis lupus*) blev officielt udryddet i Danmark i 1813 og vendte først med sikkerhed tilbage i 2012, da kadaveret fra en ulv blev fundet i Thy. Vores mål i denne opgave var at estimere antallet af ulve i Danmark og bestemme hvor fra de stammede.
Vi undersøgte 145 prøver af fækalier, spyt og hår med tre forskellige primer par, som amplificerede forskellige områder af D-Loop kontrolregionen på det mitokondrielle DNA.

To prøver blev identificeret som ulve. Da vi analyserede vores ulve prøver med sekvenser fra Århus Universitet, samt sekvenser brugt i andre undersøgelser, fandt vi fire forskellige haplotyper, hvor to er sikre. Ud fra vores resulter kan vi sige med sikkerhed at i hvert fald tre ulve har været til stede i Danmark siden 2012. Begge ulveprøver blev identificeret som en central europæisk haplotype. Bedre resultater kan opnås ved at etablere nationale guidelines. Dette vil medføre at resultater fra forskellige undersøgelser vil være mere sammenlignelig.

1. Introduction

The grey wolf *Canis lupus* was the most distributed large carnivore in Europe. The species was present in the whole continent until humans began hunting them (Aggerwal *et al* 2007, Caniglia *et al* 2013, Randi 2011, Vila *et al* 1999). It is not clear why, but the species has never been present below the equator, though it is suspected that larger predators and a lack of suitable prey may be the cause (Hein 2015). The most abundant subspecies of the grey wolf is the Eurasian wolf (*Canis lupus lupus*), ranging from Portugal to China (Hein 2015, Vila *et al* 1999). The wolf is a top predator and changes the whole ecosystem when present (Randi 2011). One of the ways wolves may affect the ecosystem is by changing the prey animal spatial behavior and thus grazing pressure in different areas (Fortin *et al* 2005). Another factor, it is the reduction of prey by wolves (e.g. deer) and thus a grazing pressure reduction overall.

The grey wolf (*Canis lupus*) was in Denmark, at least 13,000 years ago. This is confirmed by the findings of wolf archeological remains (Hein 2015). Humans arrived at approximately the same time (Gregersen 1990). The two species were after the same prey, the caribou (Gregersen 1990). In a long time the grey wolf and the human lived as rivals, as the humans were a hunter society, and the two species established themselves in Denmark. Some of the wolves were domesticated by the humans and used as hunting partners, becoming what we know today as dogs (*Canis lupus familliaris*) (Gregersen 1990, Hein 2015). This happened as early as 40.000 years ago in Europe (Callaway 2015) and approximately 15.000 years ago in Asia (Savolainen *et al* 2002).

When humans started to use cultivated fields and domesticated animals as their food source 6,000 years back, the wolf became a threat to the newly domesticated animals such as sheep and cows (Hein 2015). The domesticated animals were an easy prey for the wolf and the humans had to take some precautions to protect their new food source. Some of the dogs were then bred for a new purpose, to fight off any wolf attacks on the livestock. The wolf was no longer a rival, but a threat to the humans' new food source.

Systematic killing of the grey wolf started in the Middle Ages (Gregersen 1990, Hein 2015). A wolf tax was introduced to cover the expenses of the nobles' hunt on the wolf. Wolf hunts were arranged and a local wolf hunter was hired in many regions in Denmark (Gregersen 1990). An even more systematic hunting technique was introduced in the 17th hundred. This technique involved many men on a line that could reach as far as 50 km. The men made loud noises and forced all wolves in the covered area to flee from their dens and then a professional hunter would kill the wolves (Gregersen 1990). Many hunts were arranged and a reward was offered for every slain wolf. This intensive hunting of the wolves combined with the reduced habitat led to its extinction in Denmark in 1813 (Andersen *et al* 2015, Gregersen 1990).

The systematic hunting on the wolf in the Middle Ages, and in the time following, happened all over Europe. These hunting activities lead to the extinction of the wolf in some of the territories, but also the movement of the species to areas where it had never been settled before (Hein 2015). The period with most systematic killing in Western Europe was in the 18th and 19th centuries (Randi 2011).

A change in the environmental and climatic conditions has allowed the return of wolf's prey, larger grazing animals. Combined with legal protection of the grey wolf, the species is now spreading again throughout Europe (Caniglia *et al* 2013, Mattisson *et al* 2013, Randi 2011). The first grey wolf in Denmark was found in 2012. A dead body of a wolf-like creature was found in Thy in Jutland. Later an identification of the species was performed using a DNA-chip, which confirmed that the specimen was a grey wolf. The wolf was then tracked back to a wolf pack in Germany, the Milkeler pack Sachsen (Andersen *et al* 2015).

2. The Grey Wolf

The grey wolf occurs in all types of habitats in the Northern Hemisphere (Mech 1970, Vila *et al* 1999). This includes everything from close-knit forest to agricultural grasslands (Geffen *et al* 2004). The only types of habitats not occupied by the grey wolf include tropical rainforest (Geffen *et al* 2004), arid deserts as well as high mountain tops (Mech

1970). The wide distribution pattern suggests that geographical distances or barriers do not limit wolves in Europe as many other animals on the continent (Geffen *et al* 2004).
The limiting factors are prey availability, opportunities and area available for establishing home ranges (Mech 1970). Home ranges are determined by the availability of resources for survival and successful reproduction (Mattisson *et al* 2013).

The components of the grey wolf diet, includes roe, fallow and red deer, as well as smaller mammals and fruits (Madsen *et al* 2015, Nowak *et al* 2011), where roe deer serves as the main food source in those cases, but the diet can vary depending on the geographical distribution (Gade-Jørgensen & Stagegaard 2000, Jedrzejewski *et al* 2012, Sand *et al* 2008). Roe deer is widely distributed in Denmark and the number has only increased after farmers began growing winter crops and clearing the landscape (Olesen *et al* 2002, Wagner *et al* 2012).

2.1 The pack

Wolf populations are divided into family packs with a single breeding pair (the alphas), their off-springs, as well as unrelated individuals that are migrating among packs (Randi 2011), and adoptees (Hein 2015). Most packs consist of less than eight members, but more has been seen before (Mech 1970). The number of wolves in a family typically reflects the size of the home range and, in that respect, also the territory as well as food availability (Hein 2015). Other factors deciding pack size could be the smallest number of individuals needed to locate and kill prey, the largest number of individuals that can feed on one prey, such as a roe deer, the maximum number of individuals with which one wolf can have a social bond with and the amount of acceptable social competition (Mech 1970).

Since the grey wolf is a highly mobile animal, they have large territories and home ranges (Randi 2011). Movements during the year are determined by the migration of some prey such as caribou. The density of prey, elevation and latitude determines the wolf pack's home ranges (Mattisson *et al* 2013). In areas with high densities of prey, for example in the cultured landscape in Denmark, there is no need for large home ranges (Mech 1970). Studies have shown that home ranges, in general, are smaller in agricultural areas, because resource quality is high enough to sustain wolf population (Mattisson *et al* 2013).

2.2 The Pack Behaviour

Wolf packs' social behavior and organization is structured by a hierarchy. A well-established pack may consist of an alpha male and female, non-breeding adults (both related and unrelated to the breeding pair) each with its own ranking in the pack and some lower ranking wolves that are either outcasts or immature pups (Mech 1970, Savage & Mech 1989). The leading pair distributes work. The female primarily overlooks defenses and pups care, whereas the male overlooks foraging and travel (Mech 1999).

The lower ranking wolves submit to the dominant pair by crawling over the ground and pressing the snout to the dominants mouth. Everybody, even the unrelated individuals, help in rearing the young pups, which suggests that during the evolution of social structure the selection pressure was on pack survivability rather than the individual (Rabb *et al* 1967).

When wolf pups become sexually mature most leave their birth packs to find an unoccupied territory or to join an already existing pack. Dispersing distances depends on gender, available habitat, food and other packs (EUROBATS Secretariat 2004, Western Wildlife Outreach). Wolves of both sexes disperse, and there seem to be few consistent male-female differences in dispersal characteristics. In some regions or times, males apparently disperse farther or at a higher rate. However, at other times or places, females disperse farther on average, even though the longest-distance dispersers were males (Fritts 1983). Nevertheless, the record dispersal lengths of males and females tend to be about the same. In south-central Alaska, males dispersed at higher rate than females.

2.3 Haplotypes and subspecies in Europe

Even though wolves might disperse over great distances and are seemingly not restricted by geographical barriers (Geffen *et al* 2004), studies have shown that there are genetic different packs in Europe (Stronen *et al* 2013). The Large Carnivore Initiative for Europe has estimated approximately 10 populations: 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 (de Groot *et al* 2016).

Six subspecies of grey wolf has been proven and four more subspecies may be present but these are still debatable. *Canis lupus lupus* is the one distributed in Europe. The others are *Canis lupus campestris, Canis lupus albus, Canis lupus tundrarum, Canis lupus lycaon* and *Canis lupus nubilus (Aggerwal et al 2007)*. In Europe 27 different haplotypes of the grey wolf have been found. These can be divided into haplotypes unique for different parts of Europe, see figure 1 (Pilot *et al* 2010).

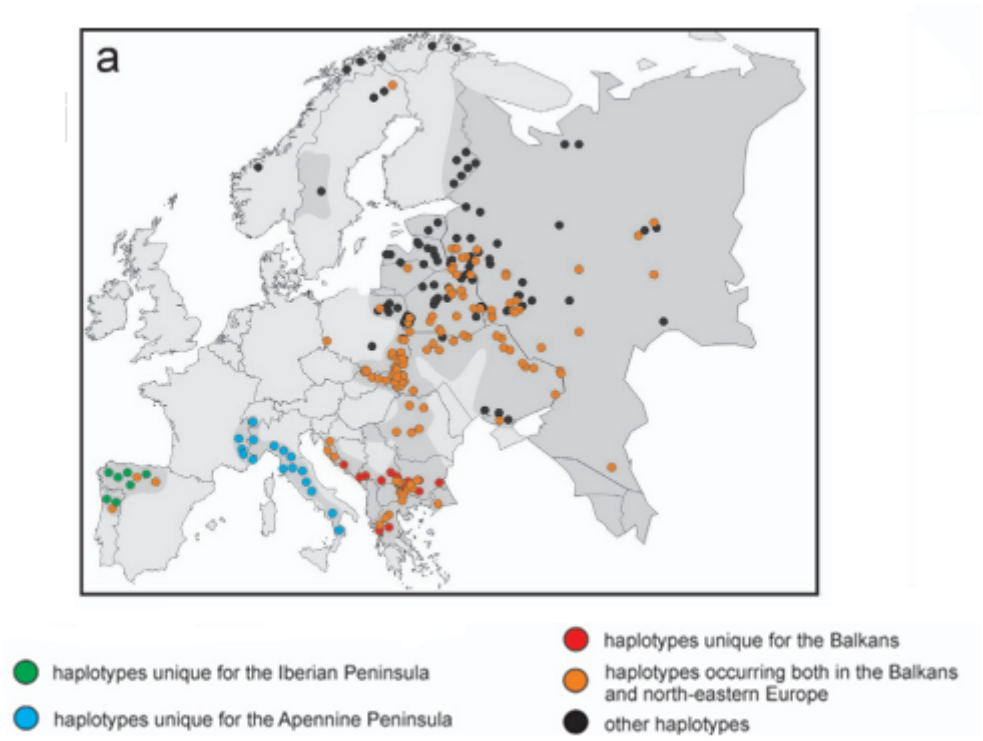

Figure 1: Map of the 27 different haplotypes registered in 2010 (Pilot *et al* 2010)

2.4 Different methods in determining size of population

Many different methods have been used to estimate the population size and to separate different individuals (de Groot *et al* 2016).

Mitochondrial DNA (mtDNA) is a method used in many studies such as in Vanbrabant *et al* (2009), Groot *et al* (2016), Aggerwal *et al* (2007) and Weber *et al* (2013). It estimates the genetic diversity in recently diverged populations (Vanbrabant *et al* 2009), both over the regional scale as well as the continental scale (Aggerwal *et al* 2007). It is suitable because of its high mutation rate and lack of recombination in the D-Loop region (de Groot *et al* 2016). Since mtDNA is based on the maternal inheritance it can give a biased view of the populations (de Groot *et al* 2016).

To supplement the mtDNA method others have used Y-chromosome markers, Single Polymorphism Nucleotides (SNPs) and microsatellites (de Groot *et al* 2016). Y-chromosome markers could give a clarification of the role each sex plays in natural processes (Sunquist *et al* 2001), for example migrations, establishing new territories as well as contributing new genes to other packs. Studies based on SNPs are becoming common (de Groot *et al* 2016). SNPs have been shown to give a distinction in spatial genetic clusters, meaning that it has become easier to distinguish between different populations, subpopulations and hybridization (de Groot *et al* 2016). Microsatellites are highly polymorphic because of the variation in the repeated units. It can be used to, like the SNPs, link populations together and distinguish populations from each other (Bruford & Wayne 1993, de Groot *et al* 2016). One of the useful things about using microsatellites is that primers developed in one species can be used in the related taxa (Bruford & Wayne 1993).

Visual methods have also been used to study wolf populations. Camera traps have been set up in Denmark at the most likely routes of the wolf and eyewitnesses can report their sightings to the organization Ulvetracking (Ulvetracking.dk 2013). The problem with these methods is that wolves and dogs can be difficult to separate especially if the picture taken has a bad quality or the wolf observed was at a long distance. The two can be easily confused in these situations and therefore the number of wolves can be overestimated. In most cases, it is difficult to determine whether the same wolf has been observed before which will lead again to an overestimation of the individuals. A way to make sure that an individual is only counted once and to establish observations and data flow all year around is to use radio collars. David E. Ausband et al used this method to observe the movements

and rendezvous sites of the grey wolf in Idaho, USA (Ausband *et al* 2010). Radio collars can also be used in determining home range size in respect to environmental and social aspects (Mattisson *et al* 2013).
When monitoring a wolf pack one can also use snow tracking during winter (Weber 2003).

2.5 The aim of our project

In this project, we aim to estimate the number grey wolves present in Denmark from 2012 to now, and from where these originated. In order to do that, we have genetically analyzed the D-Loop region on the mitochondria of 145 collected samples to identify any wolves and compare the DNA sequence with wolves from other parts of the world. In this way, we can find out where the Danish wolves originated. When starting this we suspected that only a few number of wolves were present in Denmark, because of the short period between the first wolf finding and now. We suspect that the wolves present in Denmark originate from Germany or Poland since water seems to be a barrier (Blanco *et al* 2005) and therefore it seems unlikely that the Danish wolf population stems from Sweden or Norway.

3. Method

3.1 Study sites

In 2012, a citizen science (CS) project started in Denmark for tracking the return of the wolf. This project is called Ulvetracking (Ulvetracking.dk 2013). As part of this CS, samples of suspected wolves were collected in different parts of the country. Since 2012, most part of the sampling effort has been focused in Jutland, as wolves have been visually identified in that region. The main sampling area is Midtjylland (central Jutland) with the southernmost point being Horsens. Fecal samples have also been collected outside Jutland: 3 samples on Fyn (Funen) and 1 from Northern Zealand.

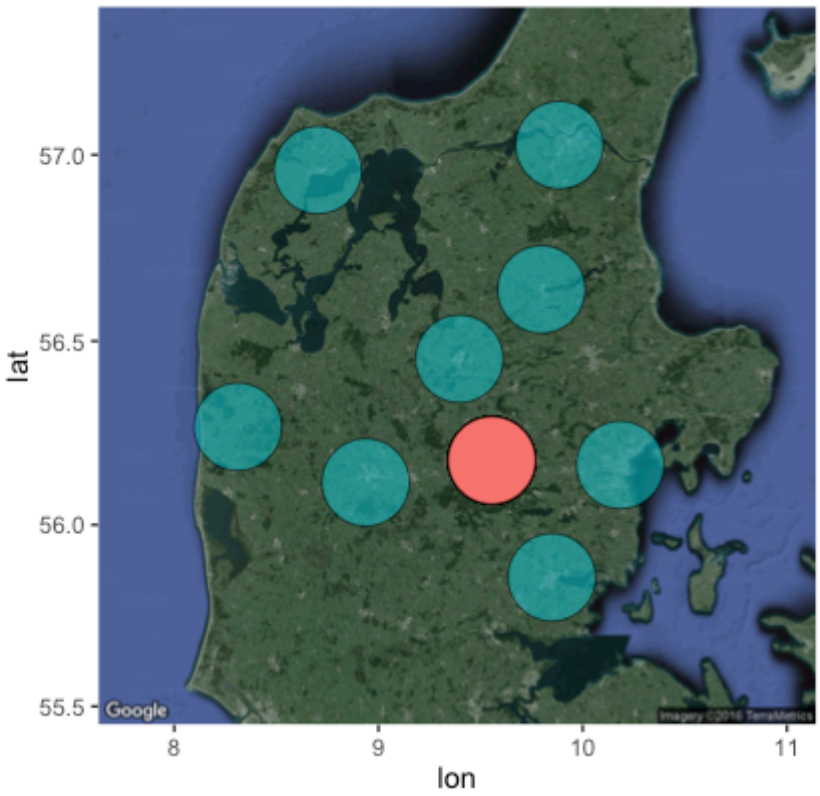

Figure 2: section of Jutland, Denmark. Dots: areas where samples were collected. Red dot: areas where samples were identified as wolf. Blue dots: areas where samples were identified as non-wolf.

3.2 Extractions

In this study, a total of 145 samples were included: one tissue sample, 12 saliva swabs, two hair/fecal samples, one hair sample, one sample of unknown origin and 128 fecal samples. Project partners performed part of the extractions in 2015, following the same procedures as described here. In 2016, 36 new samples were received and processed using different extraction methods depending on the sample type. For the fecal samples PowerFecal DNA Isolation Kit from MO BIO was used with up to 0.25 grams of starting material and following manufacturer's instructions.

For the hair, tissue and swab samples, Qiagen DNeasy Blood and Tissue was used with some modifications from the manufacturer's instructions. In the first step, double the amount of ATL was used and when extracting the hair sample 20 µL 1M DTT was added. Double the amount of ethanol and buffer AL was used in the third step and in step 7 two elutions of 70 µL AE was used instead of the described two elutions of 100 µL.

For every 8 samples a blank was included and carried on to the PCR step.

3.3 PCR and gel

To perform a species identification (ID) of the different samples, three different sets of primers that bind to the section from the control region, D-Loop on the mitochondrial DNA were used. This region of the mitochondrial genome was chosen because of its high mutation rate and non-coding properties. This means that the region has a high variability because mutations in this region do not lead to changed proteins (Aggerwal *et al* 2007, Vanbrabant *et al* 2009). This makes it easier to separate wolves from other close related canine species such as the dog, as there are more single nucleotide polymorphisms (SNPs) in this region that separates them. D-loop region has also been proved to be a good target to identify wolf prey species and other prey carnivores (i.e. foxes) (Vanbrabant *et al* 2009).

The three sets of primers are the following: primer 4 reverse/primer 4 forward (unpublished), H16498_R/L15995_F and WDLoopL/WDLoopH254 (see Table 1). The Primer 4 set was replaced with H16498_R/L15995_F for some of the samples, because the primer H16498_R/L15995_F showed a better amplification rate. To determine the sex of the wolf samples, two different primer sets, one for producing a section of the X chromosome (DBX6B and DBX6lv) and one set for producing a section of the Y chromosome (DBy7a and DBy7lv) was used (see Tabel 1 for the sequences). The five sets of primers have the following product length and sequences (Caniglia *et al* 2013, Rutkowski *et al* 2015, Seddon 2005, Weber 2003).

Tabel 1: A list of the different primers used, their names, their product length in base pairs (bp), their sequence and what they were used for.

	Bp	Sequence	Type of primer
W4F	175	5’-GGCCCATACTAACGTGGGGGT-3’	Universal
W4R	175	5’-ACTGTGGTGTCATGCATTTGGT-3’	Universal
H16498_R	370	5’-CCTGAAGTAAGAACCAGATG-3’	Universal
L15995_F	370	5’-CTCCACTATCAGCACCCAAAG-3’	Universal

WDLoopL	250	5'-TCCCTGACACCCCTACATTC-3'	For *Canis* species
WDLoopH254	250	5'-GTTTCTCGAGGCTGGTGAT-3'	For *Canis* species
DBX6B	249	5'-ATGCTGCAGT TTTTCCAGA-3'	For sex determination
DBX6lv	249	5'-AACTAAGACC CAGCGTA-3'	For sex determination
DBy7a	175	5'-GGTCCAGGAGAGGCTTTGAA-3'	For sex determination
DBy7lv	175	5'-CTTCCTTTTAAACAATGGCA-3'	For sex determination

All the samples were amplified with primer 4 or primer H/L first. For those samples showing hits for different *Canis* species or different prey species after blasting the sequences, DNA extracts were amplified with the primer WDLoop set to check whether the samples were wolf, dog or neither.

The PCR set up was as followed: 1x buffer, 2 mM MgSO4, 0,4 mM dNTPS which contained 25 mM of the four DNA bases, 0,4 µM of the two chosen primers, 0.8 mg/ml of BSA and the enzyme Taq Hifi Polymerase from the brand Invitogen. The final volume of the mixture including the DNA from the sample was 25 µL. To reach the desired concentrations, the following amount of each reagent: 2,5 µL buffer, 1 µL MgSO4, 0,1 µL dNTPs, 1 µL of the two chosen primers and BSA, 0,1 µL of the enzyme and finally 16,3 µL water was used to reach the desired volume, and 2µL of DNA from the sample was added afterwards.

The same concentration and amount of MgSO4, PCR buffer, BSA, PCR enzyme, primer 1 and primer 2 for the 3 D-Loop primers was used. For the sex determination primers a different experimental set up was used. 0,3 µM of the DBX6B and DBX6lv primer and a concentration of 0,2 µM of the DBy7a and DBy7lv primer which translates to 0,75 µL of DBX6B and DBX6lv primer and 0,5 µL of DBy7a and DBy7lv primer was used. No BSA was used. To reach the final volume, 25 µl, 16,8 µL water was added (Seddon 2005).

The PCR cycling conditions varied for the different primer pairs. The primer 4 set was run at 94 °C for 4 min, then 40 cycles of 94 °C for 30 sec, 55 °C 30 sec and 68 °C for 30 sec. Last 72 °C for 7 min and then a resting temperature at 4 °C.

The program used for the primer set H16498_R/L15995_F was 94 °C for 4 min, then 40 cycles of 94 °C for 30 sec, 50 °C 30 sec and 68 °C for 30 sec. Last 72 °C for 7 min and then a resting temperature at 4 °C (Weber 2003). Some of the samples did not work at 50 °C in the 40 cycles, so we used a temperature at 52 °C and 55 °C for some of the samples.

For the primer set WDLoopL/WDLoopH254 we ran a PCR which was 94 °C for 4 min, then 40 cycles of 94 °C for 30 sec, 55 °C 30 sec and 68 °C for 30 sec. Last 72 °C for 7 min and then a resting temperature at 4 °C. (Caniglia *et al* 2013, Rutkowski *et al* 2015).

The two sex primer sets were multiplexed in the same reaction as followed: 95 °C for 15 min and then 20 cycles of 95 °C for 30 sec, 60 °C for 40 sec with a 0.5 °C reduction of the temperature per cycle and 68 °C for 1 min. Then another 20 cycles with 95 °C for 30 sec, 50 °C for 40 sec and 68 °C for 1 min, with a final elongation at 72 °C for 15 min. and a resting temperature at 4 C (Seddon 2005).

After the PCR gel electrolysis was run to see if the PCR product had the same length as the expected product from the chosen primer set. PCR products were run through a 2% agarose gel with different ladders for each primer set. A 20 bp ladder was used for the primer 4 set, 50 bp for the H16498_R/L15995_F primer set and 50 bp when running the samples with the four sex primers. The gel electrolysis was run at 140 Volt for 30 minutes. Afterwards a picture with UV-light was taken to check the length of the samples. When the PCR products were positive and the blanks negative, samples were sent to Macrogen Europe for Sanger sequencing using the same primer pairs as for the PCRs.

The gel run with the four sex primers was analyzed and if there were two fragments, one at the length of the X chromosome product and one at the length Y chromosome product respectively, the wolf was identified as a male. If only one fragment was visible at the X chromosome product length, the wolf was identified as a female. Three replicates were made for the wolf samples.

3.4 Analysis of Macrogen results

Geneious version 7.0.6 (Kearse *et al* 2012) was used to analyze the results from Macrogen. Here the tool “map to reference” was used, which mapped the sequences from the primers, both forward and reverse, the two sequences from Macrogen, and to a Danish wolf mitochondrial genome reference (unpublished). The ends of the sequences were trimmed and mismatches were visually inspected. The forward and reserved sequences were aligned and consensus sequences were created.

3.5. Species identification

Consensus sequences were blasted in the NCBI database. When doing this BLAST algorithm searches through the DNA sequence database and find matches to the blasted DNA sequence. For our BLAST searches, we used the nucleotide database, using a 99% similarity cut off to ID our samples. To visualize the results Microsoft office excel 2013 was used to make graphs.

3.6 Phylogenetic analysis

For a better assessment of the species identification within the *Canis* genus, a phylogenetic tree was constructed to analyze the clustering of our sequences with other dog and wolf CR sequences downloaded from the NCBI. First a tree was constructed in MEGA version 6.06 (Tamura *et al* 2013) using Neighbor joining algorithm with a 1000 bootstrap. The Neighbor joining method is based on calculations of the distance between each node on the tree. The sequence alignment used had a length on 150 bp. For information on the sequences from NCBI see supplementary Table 2.
A maximum likelihood tree was also made with PhyML (Guindon *et al* 2010) online service (http://www.atgc-montpellier.fr/phyml/) and visualized with FigTree (Bandelt *et al* 1995). The tree was first calculated using PhyML with 1000 bootstrap runs under the evolutionary model HKY85. Afterwards the tree was visualized and adapted in FigTree version 1.4.2.
Three dogs from our analysis were excluded from the phylogenetic trees.

We used DNAsp Version 5.10 to obtain the haplotypes present in our wolf sequences. The length of the sequence alignment used was 151 bp and 48 sequences were used in total. This includes six wolf samples from Århus University, 39 different sequences from NCBI

(Jansson *et al* 2014, Pilot *et al* 2010) and Danish wolf (unpublished). For more information on the sequences used, see Supplementary Table 2. When calculating the haplotypes we consider gaps since these can occur in our selected sequences. Invariable sites were not considered and a Roehl Data file was generated to use in Network. The generated data file was used in Network version 5.0 (Bandelt *et al* 1995). A Median-Joining network was created for the haplotypes (figure 8).

4. Results

4.1 Species identification

For the 145 samples analyzed in this study, we were able to successfully extract and amplify our target regions from all of them. For all the samples, there was complete agreement between the forward and reverse sequences when inspected in Geneiuos version 7.0.6. No DNA was amplified from the negative controls, indicating that there was no contamination during DNA extraction or PCR set up. A list of all the amplified regions for the different samples can be found in the Supplementary Table 1.

From 145 samples we have identified 93 foxes, 9-10 deer, 2 sheep, 18 cats, and 22 *Canis* species. From the 22 *Canis* species, 20 were dogs and 2 were wolves. From four of our samples we were only able to identify down to family level, Felidae, as the same similarity was obtained in BLAST for different genus. One sample had an unclear result and it is identified as either deer or goat. Figure 3 summarizes the results from the species identification analysis.

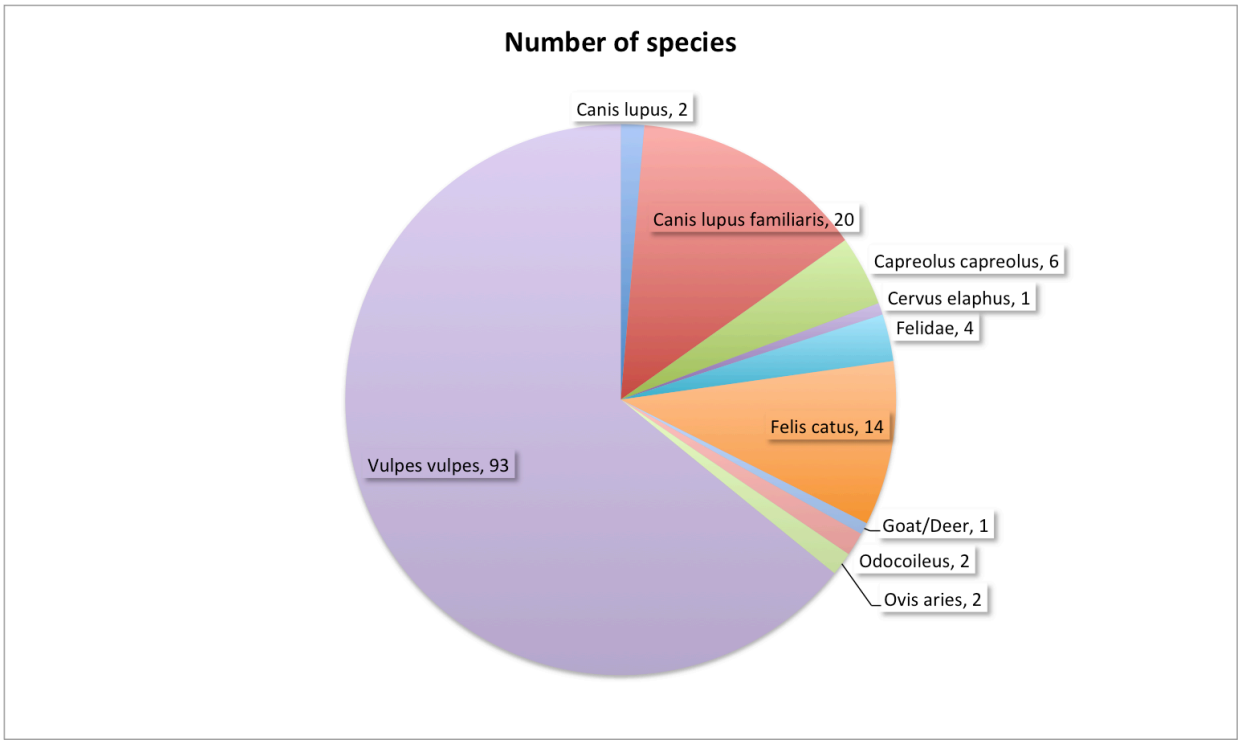

Figure 3: Number of species found in the 145 samples.

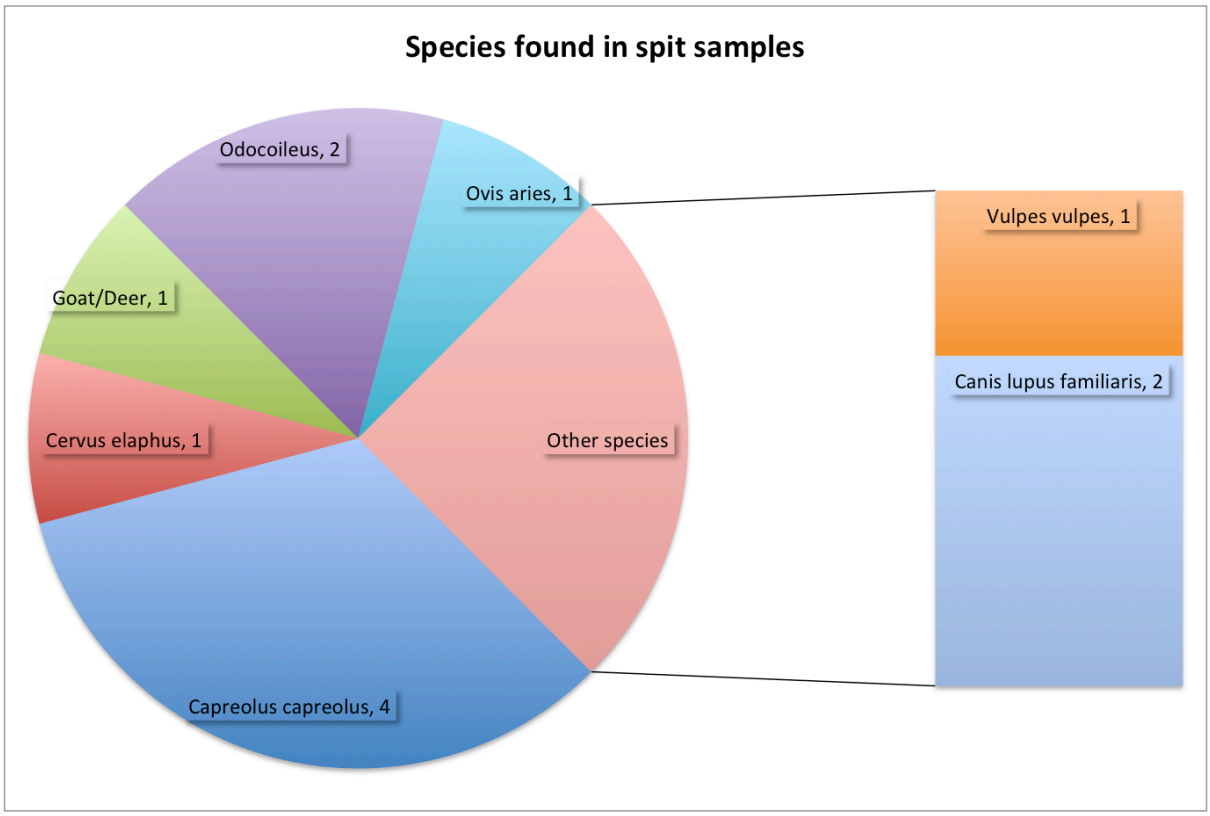

Figure 4: Species found in spit samples.

Out of the 12 spit samples, nine were prey species. This may be because the swabs had more of the prey DNA than the predator's DNA or none at all. When amplifying these with the universal primer set or with primer 4, the prey species is often amplified instead of the predator. Using the *Canis* specific primer set can counteract this. This primer should only amplify *Canis* species. However, our results show that only two dogs were identified using predator specific primers from spit samples, see figure 4.

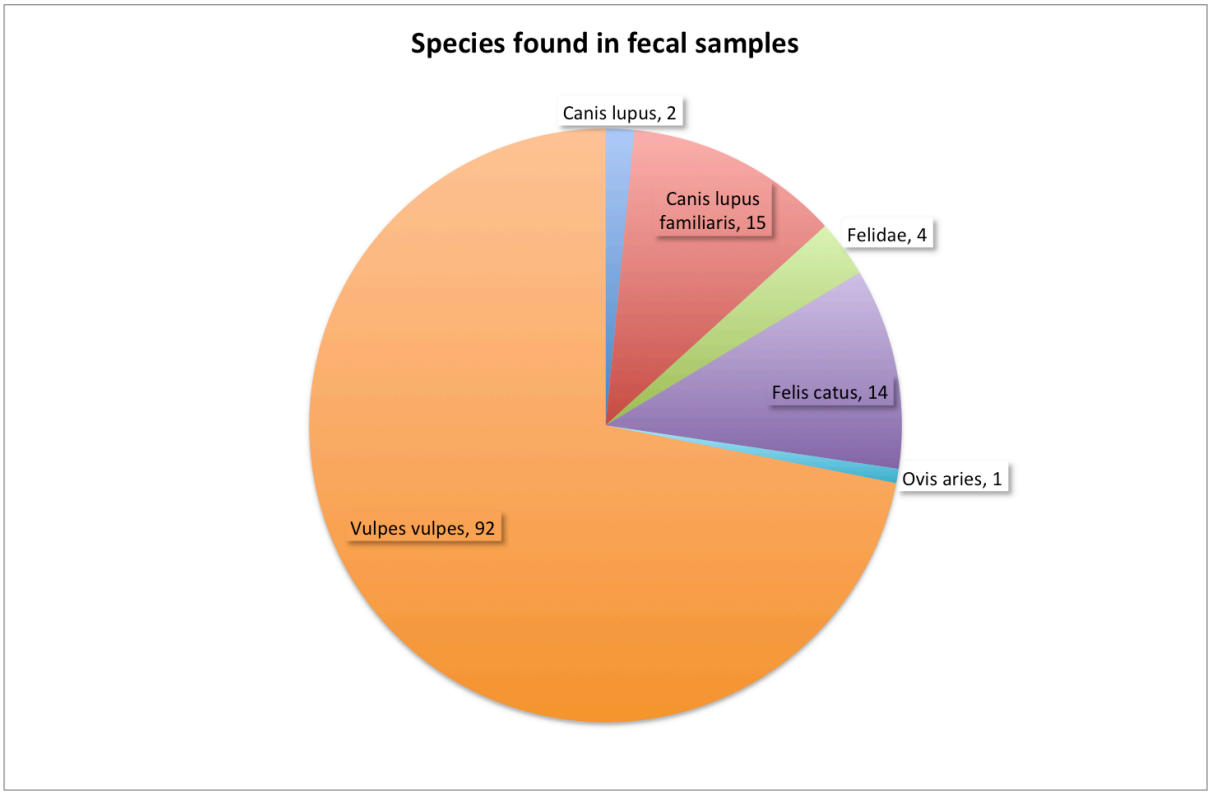

Figure 5: Species found in fecal samples.

In the fecal samples only one prey species was identified. This could be prey DNA in the predators stool or just a fecal sample from a sheep. All other fecal samples were identified as predators. Two wolves were identified from the fecal samples, see figure 5.

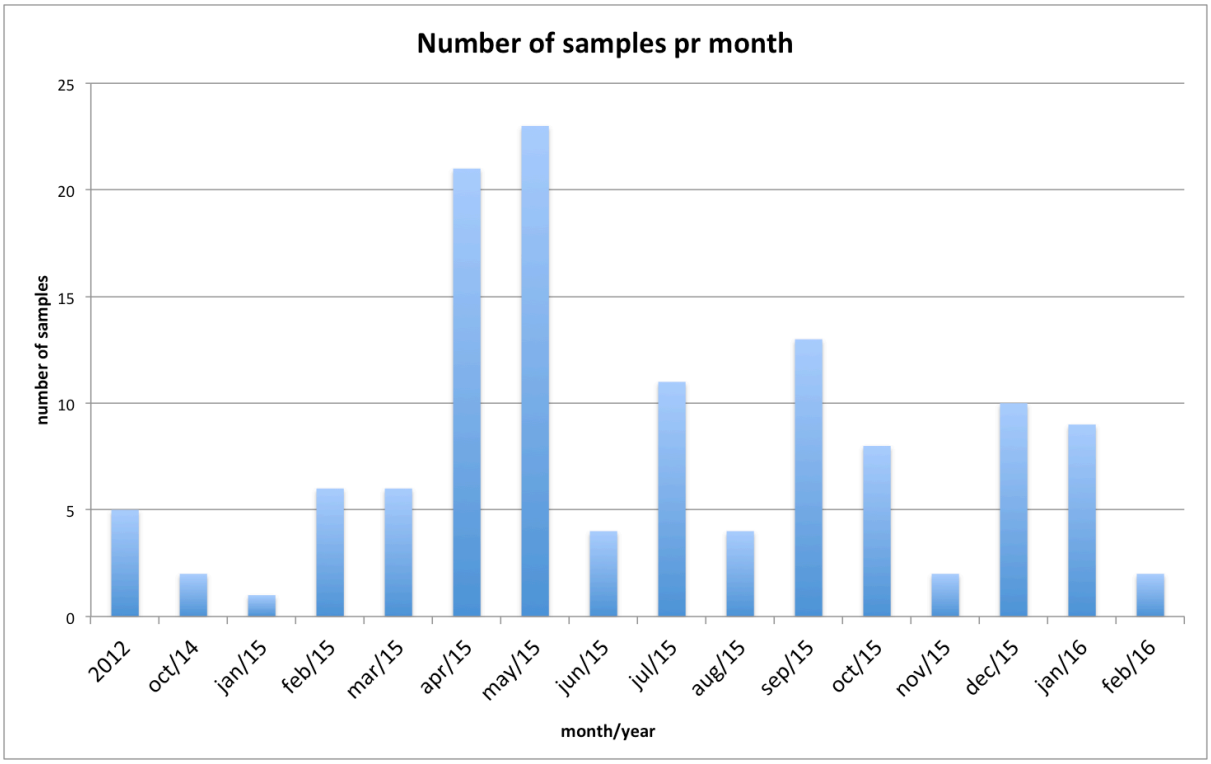

Figure 6: Number of samples collected in each month from 2012-2016.

Samples have been collected during all months in 2015 but most of the samples have been collected during April and May (see figure 6).

4.2 Phylogenetic analysis

Figure 7 shows a neighbor-joining tree for our *Canis* samples combined with other *Canis* sequences downloaded from the NCBI. Despite the low bootstrap support for some of the splits, 22% as the lowest, it is still possible to see two clear groups, a wolf cluster and a dog cluster. Our two wolf samples are all clustering together with other wolf CR sequences, while the entire dog sequences are grouped together. This supports the BLAST species identification and reflects the robustness of our results.

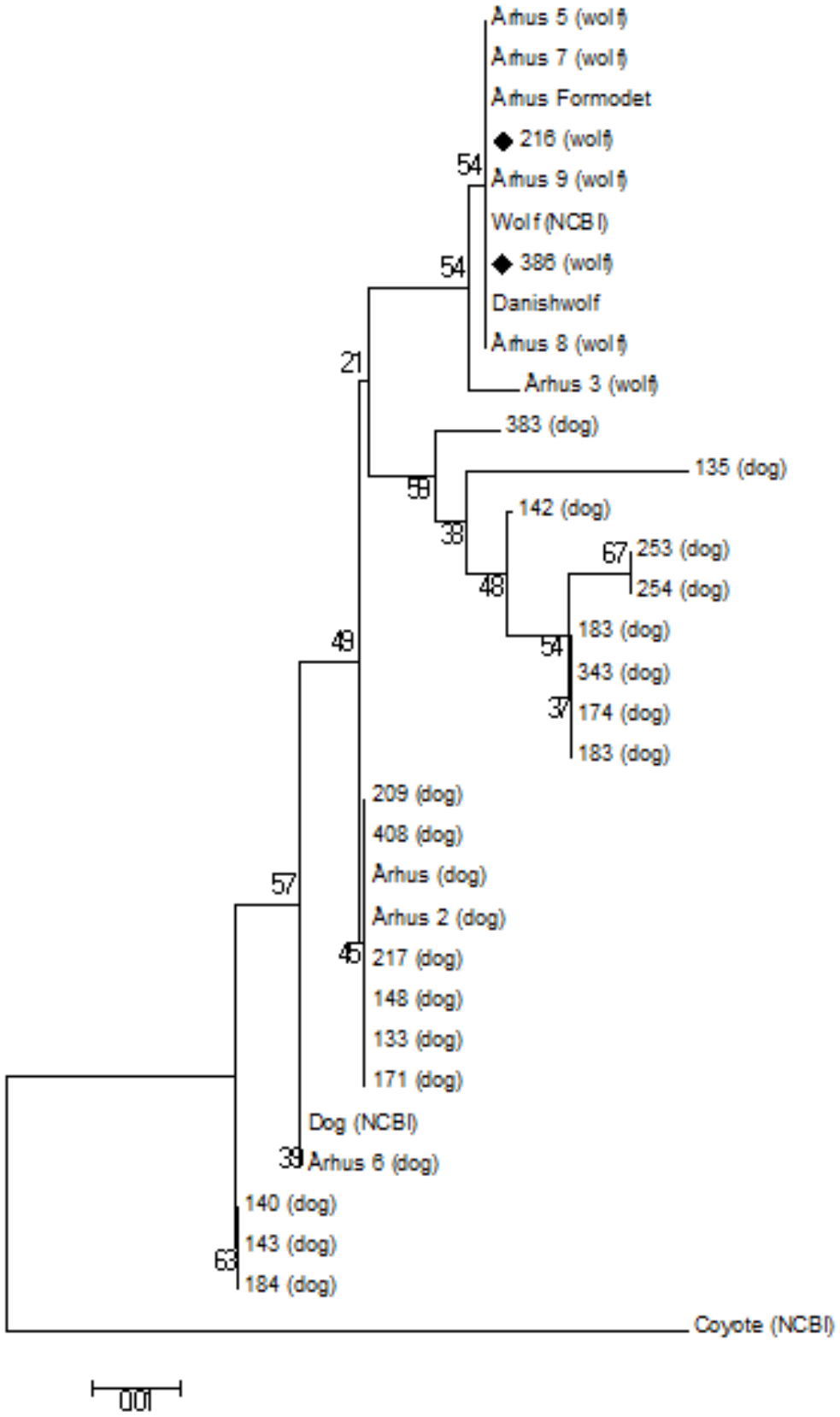

Figure 7: Neighbor joining tree made in MEGA with 1000 bootstrap runs. Samples with black diamonds: wolf samples from our analysis.

A phylogenetic tree was also done in PhyML, but no significant differences were observed. It is therefore not included in report.

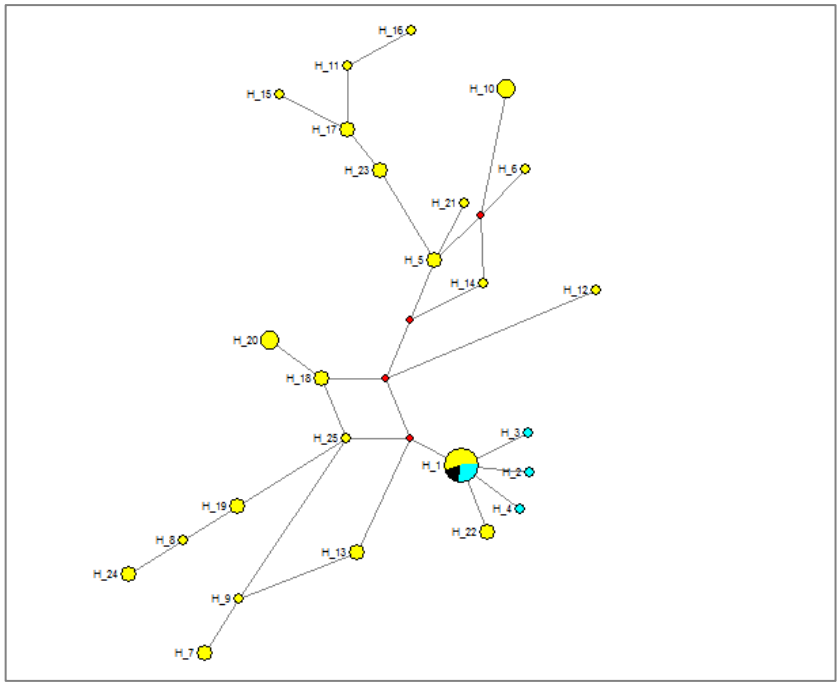

Figure 8: Network over European haplotypes for wolves. Yellow: wolf haplotypes from Jansson *et al* 2014, Pilot *et al* 2010. Black: our wolf samples. Blue: Århus' wolf samples.

Using DNAsp we have grouped our two wolf samples with six wolf sequences from Århus and 38 wolf sequences from NCBI (Jansson *et al* 2014, Pilot *et al* 2010) into 24 different haplotypes seen in figure 8. We were able to group our two samples in haplotype 1 with five sequences from Jansson *et al* (2014) and Pilot *et al* (2010), the Danish wolf (unpublished) and three from Århus. The five sequences from Jansson et al (2014) and Pilot et al (2010) are FJ978005.1, FJ978006.1, KF723520.1, KF723521.1 and KF723525.1. They stem from wolves found in Poland, Latvia, Estonia, Belarus, Ukraine, Russia, Sweden, Norway and Finland. Three of Århus' samples have slightly different haplotypes, haplotype 2-4, but only with one base pair in difference from haplotype 1. Overall Århus' samples were grouped in four different haplotypes, haplotype 1-4.

Our network shows that the haplotype, which our wolf samples are grouped in, is most related to haplotype 2-4, which is all samples from Århus, and haplotype 22, which includes FJ978022.1 from Belarus and Russia and KF723526.1 from Finland. Furthermore haplotype 13 and 25 are both one link from haplotype 1. Haplotype 13 includes

FJ978024.1 and FJ978023.1 from Bulgaria, Greece and former Yugoslavia. Haplotype 25 includes FJ978034.1 from Russia.

4.3 Sex identification

The two wolf samples were identified as male. The X-chromosome fragment was not amplified, but the Y fragment was clearly visible in all replicates. The X-chromosome fragment is difficult to amplify and it is therefore acceptable that only the Y fragment is visible (Seddon 2005).

5. Discussion:

5.1 Our results

Since the first ID of a wolf in Denmark in 2012 (Madsen *et al* 2015), a lot of work has been done to study the number of wolf individuals in the territory and, to some extent, start a conservation program. Considering the pack structure and movement of the species (Hein 2015, Mattisson *et al* 2013, Mech 1970, Randi 2011, Savage & Mech 1989), and the short time since the first wolf was spotted in Denmark (Madsen *et al* 2015), our expectations were to identify 1 to 6 wolves in Denmark. As mentioned above we found two samples with wolf DNA present in a pool of 145 samples, however, it is not clear yet whether our two wolf samples are from the same individual as they have been identified as the same haplotype (figure 8). Genotyping of the samples will be done in a next step. Three of the wolf samples from Århus were identified as having three different haplotypes from our wolf samples, therefore it is possible to estimate that at least five wolves have been in Denmark since 2012 until now, including the Thy wolf (Andersen *et al* 2015). These results are in line with other studies of the wolf in Europe. It is possible that more wolves are present, thus an increased sampling effort is necessary for a better understanding of the individual number. A more precise estimate of number of individuals could also be reached if some nuclear makers were genotyped or if full mitochondrial genomes could be generated from each of the samples.

5.2 Our methods

The wolf samples were collected in 2015 and 2016 in close proximity to Silkeborg, see figure 2. We have tried to reduce a false negative of wolf by using different primers; two for a general identification of species, primer set 4 and the universal primer set, and one for any *Canis* species.

We tried to determine the size of the wolf population in Denmark by using fecal and spit samples. Fecal samples proved to be the most accurate sampling method, though it has some difficulties. The problem with fecal samples is the degradation from the digestions, for being out in a field, rain and high temperatures.
Most of our samples have been collected in April and May (figure 6) and the temperatures are fairly low in most days of these months, though there is a possibility for high temperatures during this time and this may affect the fecal samples because of a higher rate of degradation (Lucchini *et al* 2002). Samples had a higher possibility for a successful amplification when the samples were relatively fresh (Lucchini *et al* 2002, Piggott 2004). A study was conducted to determine whether rain had an effect on the quality of the fecal samples. Samples that were collected during a dry period showed a higher number of successfully amplified samples in comparison to samples collected in a wet period (Farrell *et al* 2000). The average precipitation of rain was in April 2015 27mm (Cappelen 2015a) and in May 2015 86mm (Cappelen 2015b). A higher level of rain, as seen in May, could have resulted in a higher rate of degradation of the samples collected in this period.

When the DNA is fragmented as seen in fecal samples, specific primers should be used. The primer set used should only target short areas of the mtDNA so that even a small piece of DNA from a species can be identified. To amplify a longer part of the DNA, more sets of primers can be used, all targeting short areas (Andersen & Madsen 2013, Fernandes *et al* 2008). The fragments amplified can be combined and a longer piece of the DNA from the sample described. If a primer set, which target site is too long, is used, the amplification can be difficult. Very little DNA may be amplified or none.

When working with the spit samples, the amplification of the prey species instead of the predator from swab extracted DNA has been a challenged for us. In a study by Harms et

al. (2015), the rate of error in identification of wolves using spit samples was investigated. The success rate dropped greatly, from over 83% to less than 50%, if the prey was swabbed for spit at least 48 hours after instead of 1 hour or 24 hours after the bite. This may be the cause of the low rate of predators found from spit samples (figure 4). Only two predator species were found, but only one species is likely to have killed the deer. This suggests that scavengers DNA may also be sampled from the carcass. Another reason for the difficulties determining the predator species could be because of the lack of training of the people collecting the spit samples. It might be necessary to establish some specific guidelines for spit collection, regarding how to do it and when to do it. The use of target specific primers improved our results, however it is not clear for us that this yields the best results. A way to improve the identification of predators could be to optimize the PCR program for the spit samples, however it is not clear whether anymore wolves would have been found in this way, but it could increase the success rate of finding a predator.

5.3 Sequence length

When analyzing the haplotype and making the tree the alignment was only 150 bp and 151 bp. We had to cut it so it would fit with the samples from Århus. The shortened sequences means that the trees made have poorer bootstrap quality. International guidelines in wildlife forensic and conservation could help resolve this problem. If there was a defined minimum length the analyzed sequence should have, it would make the results from different project comparable. The short sequences may also cause miss-identification of the samples so with a guideline, more reliable results could be made.

5.4 Sampling

As seen in figure 3, most part of the samples were identified as non-target species, such as foxes or cats. A critic point in this regard is the sampling method. The samples used in our study have been collected volunteers under the Citizen Science project Ulvetracking (Ulvetracking.dk 2013). Citizen Science initiatives are a great way of engaging a general public in research. However, due to the history of DNA identification of suspected wolf fecal samples in Denmark the training in proper wolf sample identification and collection has been insufficient. The numbers of our study suggest that education and training of the public using proper material would increase the collection efficiency and reduce the time

spend in the molecular ID process. As we can see there has already been an improvement in the late 2015 and early 2016, where one wolf was found in 36 newly collected samples compared to 1 wolf in 109 samples in the old samples. This might reflect a chance in the sampling strategy. It is difficult to tell the difference in fecal samples without training, and the age of the fecal sample is unknown which will affect the quality of the DNA in the samples (Lucchini *et al* 2002). A higher rate and better quality of *Canis* fecal samples may be obtained with more training. It is not clear whether the spit samples have been made in the correct way and this could be the reason most of them only resulted in the specimen the sample was taken from.

5.5 Århus University results

Initially when Århus University conducted their research regarding the population size of the wolves in Denmark they got volunteers to collect and send in samples via Ulvetracking. This meant that the search area was larger and the possibility of finding evidence of the wolves' presence was bigger. In 2015, Aarhus University presented some results indicating that there were 20 to 23 wolf individuals in Denmark (Andersen *et al* 2015, Jensen *et al* 2015, Madsen *et al* 2015). These results have been much disputed, and there are several considerations to reflect on.

Århus used the Qiagen DNA Investigator Kit to extract DNA from both fecal and spit samples (Andersen *et al* 2015). This kit is, according to Qiagen's webpage, mostly used DNA extractions from spit, dried blood and so forth, but it is unclear whether it is suitable for DNA extractions from fecal samples. The PCR protocol used in their amplification is not clearly stated in the three articles published, except for a 240 bp long DNA-marker or primer that was designed using available sequences on the NCBI. This marker would be able to distinguish wolf from dog (Andersen & Madsen 2013).

The final news about their numbers is a report send by their German collaborators from Senckenberg to Naturstyrelsen. Fifty samples were sent to Germany to validate their results and the Germans found that only six samples could be identified as wolves and out of the six samples only 3 individuals could be determined (Rahbek & Hansen 2016), which meant that only 4 different individuals, including the Thy wolf, had been observed in Denmark.

In June 2016 Århus University and DCE (Nationalt Center for Miljø og Energi) withdrew their statement on the number of wolves in Denmark. The new statement is that four individuals have been identified in Denmark (Ramskov 2016, Skaaning 2016).

5.6 The future for the grey wolf in Denmark

The diet of the grey wolf consists mostly of roe deer, *Capreolus capreolus*, and red deer, *Cervus elaphus*. Supplementary prey is fallow deer, *Dama dama* (Nowak et al 2011). The roe deer has good conditions in Denmark. It prefers open areas and the modern landscapes in Denmark with a lot of fields suits the roe deer (Olesen *et al* 2002). The grey wolf is an opportunistic predator and can adapt to local conditions evolving into specialist on feeding on the prey that are available in the area they live (Randi 2011) and the good conditions for roe deer in Denmark, makes it a suitable area for the grey wolf to live.

There is no clear number of wolves, which the Danish nature could support. It is hard to determine because of the flexible home range of wolves (Mattisson *et al* 2013). It could be predicted that the wolves do not need large home ranges in Denmark, because of the high density of prey (Mech 1970).

The human population could also limit the wolves' habitat possibilities. Highways are a barrier for the wolf, but many of these have passageways for wildlife in Denmark. It is the roads without the barrier that is the most dangerous for the wolves (Hein 2015).
In Jutland in Denmark the population density of humans is 73 humans per km^2. This is the same density as in the Polish province Lubusz were big parts of the Polish wolf population lives. The wolf thrives in other countries with a higher or same human density as found in Jutland (Hein 2015). The wolf's spreading pattern is limited by the long coastline and many islands found in Denmark (Hein 2015).

Throughout the history the wolf has been described as a vicious beast with a thirst for human blood. They are portrayed as evil murderers who hunt humans (Gregersen 1990). Many of the histories regarding attacks on human are either exaggerated or caused by rabies (Gregersen 1990, Hein 2015). Some attacks have happened through the modern

years, but many can be explained by wolves who were too used to humans, a serious lack of prey or unsupervised children (Hein 2015).

In Denmark a questionnaire was conducted from 2007 to 2008. 62% of the participants is concerned for their family's safety if the wolf returns, and 48% of the participants thought that the wolf had no right to return to Denmark and should be kept out (Hein 2015). Many Danes are concerned for their livestock as most of the livestock predation is attributed to the wolf in Europe (Caniglia *et al* 2013). Since dog and wolf kills are hard to distinguish from each other, illegal poaching of the wolves has become a reality in different parts of Europe (Caniglia *et al* 2013), even though free-ranging dog may be the cause (Echegaray & Vilà 2009, Hein 2015, Randi 2011).

Another problem about the false identification of the actual predator of the kill is the compensation cost in some European countries (Echegaray & Vilà 2009). Compensation fees can be taken advantage of if the real species is not determined and this might push managers and politicians to start control plans and legal killings (Caniglia *et al* 2013) even though the wolves are not the perpetrator in all cases.
Different conservation efforts have been made to ensure the wolves return or progress in the world. The minimum number of wolves is monitored using different methods such as snow tracking, molecular tracking, such as analysis of fecal and spit samples, diet analysis and capture recapture if possible. The capture recapture method is especially good when wanting to know the survival rates and home ranges (Weber 2003).

5.7 Conservation efforts

The re-colonisation have proven to involve some problems with the human population living near the areas, where wolf tracks have been found.
Take for example the re-colonisation of wolves in the Alps where there has been proven to be some problems regarding livestock concerns from farmers. This means that changes in e.g., sheep farming needs to be reevaluated to ensure that wolves do not get into the enclosures or that they keep away by using shepherds or guarding dogs (Weber 2003).

A conservation plan in Denmark would be to make sure that information about the wolf's behavior and habits become available for the public and ensure that it is made clear that the wolf do not pose any or a very small threat to the human populations living near them. Another idea is to explain the prosperous impact the wolf is going to have on the ecosystem in Denmark (Fortin *et al* 2005, Hein 2015). The impact is of course not going to be as big as in the Yellowstone National Park, but to some degree is will happen.

Since there is still some debate about how many wolves are present in Denmark a way to monitor them is by using methods like mtDNA markers all year and snow tracking during the winter. We do not know when we get a definitive number on the population in Denmark, but when we do it will be easier to make a plan to control and regulate the population. A possibility is to agree to controlled hunting. This will hopefully also ensure that illegal hunting will not happen (Hein 2015), and if it does the government should consider issuing fines. Controlled hunt of the wolves will also make sure that wolves do not get too comfortable with humans.

To prepare for the wolves return farmers should consider installing electric fences or other types of sturdier fencing to protect the livestock, especially free grazing sheep without any other protection.

6. Conclusion:

In our study, we examined the control region, D-Loop, on the mitochondrial DNA of 145 different samples and identified two samples with positive wolf DNA. These were determined to be in the same haplotype as some samples from Århus University as well as sequences from Finland, Russia, Belarus, Poland, Ukraine, Norway, Sweden, Latvia and Estonia which is to be expected as no German haplotypes are currently available in Genbank. Furthermore three samples from Århus University was determined to have different sub-haplotypes. Two of the Århus University samples had a gap or unidentified base in their DNA sequence and therefore two of the four possible haplotypes are not certain. This suggests that at least three different individuals have been present in Denmark since 2012. It is possible that more individuals are present, but a more thorough study needs to be conducted to fully estimate the population size. Both of our samples

were identified as males. We can conclude that our samples stem from a Central European haplotype.
We expected to find 1-6 wolves and our results confirm our expectations though it is not clear if this is the final number of wolves present from 2012 to now.

When conducting the species identification fecal samples seemed to have worked the best. Spit samples can also be used, but with some precautions. A better method for this needs to be investigated. With better guidelines a higher rate of *Canis* species is possible to achieve, though some improvements are already visible. For the samples from different studies can be comparable, an international guideline needs to be established.

When a final estimate of the wolf population in Denmark is made, it may be necessary to make a conservation plan for the wolf. The public needs information on the wolf, and farmers need to prepare for the possibility of future livestock killings.

7. References

Aggerwal RK, Kivisild T, Ramadevi J, Singh L. 2007. Mitochondrial DNA coding region sequences support the phylogenetic distinction of two Indian wolf species. *J Zool Syst Evol Res* 45: 163-72

Andersen LW, Elmeros M, Sunde P, Olsen K, Vedel-Smith C, et al. 2015. DNA-baseret bestandsovervågning afslører ulve (*Canis lupus)* i Danmark. *Flora of Fauna* 121: 60-64

Andersen LW, Madsen AB. 2013. Notat vedr. DNA analyse af spyt-skrab fra tre nedlagte får, DCE - Århus Universitet

Ausband D, Mitchell MS, Doherty KE, Holyan J. 2010. Surveying Predicted Rendezvous Sites to Monitor Gray Wolf Populations. *Journal of Wildlife Management* 74: 1043-49

Bandelt HJ, Forster P, Sykes BC, Richards MB. 1995. Mitochondrial portraits of human populations using median networks. *Genetics* 141: 753

Blanco JC, Cortés Y, Virgós E. 2005. Wolf responds to two kinds of barriers in an agricultural habitat in Spain. *Can. J. Zool.* 83: 312-23

Bruford MW, Wayne RK. 1993. Microsatellites and their application to population genetic studies. *Current Opinion in Genetics and Development* 3: 939-43

Callaway E. 2015. Ancient wolf genome puches back dawn of the dog. In *Nature*: Nature

Caniglia R, Fabbri E, Mastrogiuseppe L, Randi E. 2013. Who is who? Identification of livestock predators using forensic genetic approaches. *Forensic sci. Int. Genet* 7: 397-404

Cappelen J. 2015a. Vejret i Danmark - april 2015. DMI.dk: DMI

Cappelen J. 2015b. Vejret i Danmark - maj 2015. DMI.dk: DMI

Chepko-Sade BD, Halpin ZT. 1987. *Mammalian Dispersal Patterns - The effect of social structure on population genetics*. pp. 1-323. The University of Chicago Press. 323 pp.

de Groot GA, Nowak C, Skrbinšek T, Andersen LW, Aspi J, et al. 2016. Decades of population genetic research reveal the need for harmonization of molecular markers: the grey wolf *Canis lupus* as a case study. *Mammal Review* 46: 44-59

Echegaray J, Vilà C. 2009. Noninvasive monitoring of wolves at the edge of their distribution and the cost of their conservation. *Animal conservation* 13: 167-71

EUROBATS Secretariat. 2004. EUROBATS: The Agreement on the Conservation of Populations of European Bats. EUROBATS

Farrell LE, Roman J, Sunquist ME. 2000. Dietary separation of sympatric carnivores identified by molecular analysis of scats. *Molecular Ecology* 9: 1583-90

Fernandes CA, Ginja C, Pereira I, Tenreio R, Bruford MW, Santos-Reis M. 2008. Species-specific mitochondrial DNA markers for identification of non-invasive samples from sympatric canivores in the Iberian Peninsula. *Conserv Genet* 9: 681-90

Fortin D, Beyer HL, Boyce MS, Smith DW, Duchesne T, Mao JS. 2005. Wolves influence elk movements: behavior shapes a trophic cascade in Yellowstone National Park. *Ecology* 86: 1320-31

Geffen E, Anderson MJ, Wayne RK. 2004. Climate and habitat barriers to dispersal in highly mobile grey wolf *Molecular Ecology* 13: 2481-90

Gregersen H. 1990. *Ulveliv - ulvens historie i Danmark*. pp. 1-52. Tema Bogforlag. 52 pp.

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* 59: 307-21

Hein N. 2015. *Ulv! Dyret - Myten - Fremtiden*. pp. 1-182. Gyldendal. 182 pp.

Jansson E, Harmoinen J, Ruokonen M, Aspi J. 2014. Living on the edge: reconstructing the genetic history of the Finnish wolf population. *MBC evolutionary biology* 14: 1-20

Jensen TS, Olsen K, Sunde P, Vedel-Smith C, Madsen AB, Andersen LW. 2015. Genindvandring af ulven (*Canis lupus*) i Danmark. *Flora of Fauna* 121: 8

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, et al. 2012. Geneious Basic: An intergrated and extendable desktop software platform for the organization and analysis of sequenced data. *Bioinformatics applications note* 28: 1647-49

Lucchini V, Fabbri E, Marruco F, Recci S, Boitani L, Randi E. 2002. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the Western Italian Alps. *Molecular Ecology* 11: 857-68

Madsen AB, Elmeros M, Andersen LW, Nørgaard LS, Mikkelsen DMG, et al. 2015. De første analyser af ulvens (*Canis lupus*) føde i Danmark. *Flora of Fauna* 121: 55-59

Mattisson J, Sand H, Wabakken P, Gervasi V, Liberg O, et al. 2013. Home range size variation in a recovering wolf population: evaluating the effect of environmental, demographic, and social factors. *Oecologia* 173: 813-25

Mech LD. 1970. *The Wolf: The ecology and the behavior of an endangered species*. pp. 1-384. NHP. 384 pp.

Nowak S, Myslajek RW, Klosinska A, Gabrys G. 2011. Diet and prey selection of wolves (*Canis lupus*) recolonising Western and Cental Poland. *Mammal Biology* 76: 709-15

Olesen CR, Asferg T, Forchhammer MC. 2002. Rådyret - fåtallig til almindelig, Miljø ministeriet

Piggott M. 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping og faecal DNA. *Wildlife reseach* 31: 485-93

Pilot M, Branicki W, Jedrzejewski W, Goszczynski J, Jedrzejewska B, et al. 2010. Phylogeographic history of grey wolves in Europe. *BMC evolutionary biology* 10: 1-11

Qiagen. -. QIAamp DNA Investigator Kit. qiagen.com: Qiagen

Rabb GB, Woolpy JH, Ginsburg BE. 1967. Social relationships in a group of captive wolves. *American Zoologist* 7: 305-11
Rahbek C, Hansen AJ. 2016. Statur for ulv - tyskerne har nu meldt ud, hvad de fandt i DCE's prøver. ulvetracking.dk: Ulvetraking
Ramskov J. 2016. Sådan blev 40 ulve til 23 for at ende med 4. In *Ingeniøren*. Ing.dk
Randi E. 2011. Genetics and conservation of wolves *Canis lupus* in Europe. *Mammal Review* 41: 99-111
Rutkowski R, Krofel M, Giannatos G, Cirovic D, Männil P, et al. 2015. A European Concern? Genetic structure and expansion of Golden Jackal (*Canis aureus*) in Europe and the Caucasus. *PLoS ONE* 10: 1-22
Savage C, Mech LD. 1989. *Wolves*. pp. 1-159. Robert Hale. 159 pp.
Savolainen P, Zhang Y, Luo J, Lundeberg J, Leitner T. 2002. Genetic evidence for an East Asian origin of domestic dogs. *Science* 298: 1610-13
Seddon JM. 2005. Canid-specific primers for molecular sexing using tissue or non-invasive samples. *Conservation Genetics* 6: 147-49
Skaaning J. 2016. Fra 40 til fire: Forskere trækker ulvetal i Danmark tilbage. dr.dk
Stronen AV, Jedrzejewska B, Pertoldi C, Demontis D, Randi E, et al. 2013. North-south differentiation and a region of high diversity in European wolves (*Canis lupus*). *PLoS ONE* 8: 1-9
Sunquist AK, Ellegren H, Olivier M, Vila C. 2001. Y chromosome haplotyoing in Scandinavian wolves (*Canis lupus*) based om microsatellite markers. *Molecular Ecology* 10: 1959-66
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6. *Mol. Biol. Evol.* 30: 2725-29
Ulvetracking.dk. 2013. Velkommen til Ulvetracking Danmark. Ulvetracking.dk
Vanbrabant J, Goddeeres B, Martins K, Schön I. 2009. Development of primers for amplification and further research on the D-Loop control region for the Common Spadefoot toad, Pelobates fuscus (Amphibia). *Conserv Genet. Resour.* 1: 495-99
Vila C, Amorim IR, Leonard JA, Posada D, Castroviejo J, et al. 1999. Mitochindrial DNA phylogeography and population history of the grey wolf *Canis lupus*. *Molecular Ecology* 8: 2089-103
Weber JM. 2003. Wolf monotoring in the Alps, KORA

8. Supplementary Tables

8.1 Supplementary Table 1

Primers used on samples

CGG-number	Sequences					
	Primer 2	Primer 3	Primer 4	Primer 5	WDLoop	Uni primer
CGG_6_000133						
CGG_6_000134						
CGG_6_000135						

CGG_6_000137						
CGG_6_000140						
CGG_6_000142						
CGG_6_000143						
CGG_6_000144						
CGG_6_000145						
CGG_6_000147						
CGG_6_000148						
CGG_6_000149						
CGG_6_000151						
CGG_6_000153						
CGG_6_000154						
CGG_6_000160						
CGG_6_000162						
CGG_6_000167						
CGG_6_000171						
CGG_6_000174						
CGG_6_000177						
CGG_6_000178						
CGG_6_000179						
CGG_6_000180						
CGG_6_000182						
CGG_6_000183						
CGG_6_000184						
CGG_6_000187						
CGG_6_000188						
CGG_6_000196						
CGG_6_000197						
CGG_6_000198						
CGG_6_000199						
CGG_6_000200						
CGG_6_000203						
CGG_6_000204						
CGG_6_000207						
CGG_6_000208						
CGG_6_000209						
CGG_6_000210						
CGG_6_000212						
CGG_6_000215						
CGG_6_000216						
CGG_6_000217						

CGG_6_000226						
CGG_6_000230						
CGG_6_000232						
CGG_6_000234						
CGG_6_000236						
CGG_6_000237						
CGG_6_000240						
CGG_6_000241						
CGG_6_000242						
CGG_6_000243						
CGG_6_000246						
CGG_6_000247						
CGG_6_000248						
CGG_6_000250						
CGG_6_000251						
CGG_6_000252						
CGG_6_000253						
CGG_6_000254						
CGG_6_000255						
CGG_6_000256						
CGG_6_000257						
CGG_6_000258						
CGG_6_000259						
CGG_6_000260						
CGG_6_000261						
CGG_6_000262						
CGG_6_000263						
CGG_6_000264						
CGG_6_000265						
CGG_6_000266						
CGG_6_000267						
CGG_6_000268						
CGG_6_000269						
CGG_6_000270						
CGG_6_000271						
CGG_6_000273						
CGG_6_000274						
CGG_6_000294						
CGG_6_000307						
CGG_6_000308						
CGG_6_000309						

CGG_6_000310						
CGG_6_000319						
CGG_6_000320						
CGG_6_000323						
CGG_6_000325						
CGG_6_000327						
CGG_6_000331						
CGG_6_000333						
CGG_6_000334						
CGG_6_000337						
CGG_6_000338						
CGG_6_000341						
CGG_6_000342						
CGG_6_000343						
CGG_6_000345						
CGG_6_000346						
CGG_6_000348						
CGG_6_000349						
CGG_6_000351						
CGG_6_000352						
CGG_6_000353						
CGG_6_000354						
CGG_6_000364						
CGG_6_000365						
CGG_6_000366						
CGG_6_000367						
CGG_6_000368						
CGG_6_000406						
CGG_6_000371						
CGG_6_000372						
CGG_6_000373						
CGG_6_000374						
CGG_6_000375						
CGG_6_000376						
CGG_6_000377						
CGG_6_000378						
CGG_6_000379						
CGG_6_000380						
CGG_6_000381						
CGG_6_000382						
CGG_6_000383						

CGG_6_000384						
CGG_6_000385						
CGG_6_000386						
CGG_6_000387						
CGG_6_000388						
CGG_6_000389						
CGG_6_000390						
CGG_6_000391						
CGG_6_000392						
CGG_6_000393						
CGG_6_000394						
CGG_6_000395						
CGG_6_000396						
CGG_6_000397						
CGG_6_000398						
CGG_6_000399						
CGG_6_000400						
CGG_6_000401						
CGG_6_000402						
CGG_6_000403						
CGG_6_000404						
CGG_6_000405						
(CGG_6_000408)						

8.2 Supplementary Table 2

Sequences used in DNAsp, MEGA and PhyML, and their origin

Accession nr.	Reference	Length (bp)	Used Names	Used in program
FJ978035.1	Pilot et al. (2010)	659		DNAsp
FJ978034.1	Pilot et al. (2010)	658		DNAsp
FJ978033.1	Pilot et al. (2010)	659		DNAsp
FJ978032.1	Pilot et al. (2010)	658		DNAsp
FJ978031.1	Pilot et al. (2010)	658		DNAsp
FJ978030.1	Pilot et al. (2010)	659		DNAsp
FJ978029.1	Pilot et al. (2010)	659		DNAsp
FJ978028.1	Pilot et al. (2010)	658		DNAsp
FJ978027.1	Pilot et al. (2010)	658		DNAsp
FJ978026.1	Pilot et al. (2010)	658		DNAsp
FJ978025.1	Pilot et al. (2010)	658		DNAsp
FJ978024.1	Pilot et al. (2010)	658		DNAsp

FJ978023.1	Pilot et al. (2010)	658		DNAsp
FJ978022.1	Pilot et al. (2010)	658		DNAsp
FJ978021.1	Pilot et al. (2010)	658		DNAsp
FJ978020.1	Pilot et al. (2010)	658		DNAsp
FJ978019.1	Pilot et al. (2010)	658		DNAsp
FJ978018.1	Pilot et al. (2010)	659		DNAsp
FJ978017.1	Pilot et al. (2010)	658		DNAsp
FJ978016.1	Pilot et al. (2010)	658		DNAsp
FJ978015.1	Pilot et al. (2010)	658		DNAsp
FJ978014.1	Pilot et al. (2010)	659		DNAsp
FJ978013.1	Pilot et al. (2010)	660		DNAsp
FJ978012.1	Pilot et al. (2010)	658		DNAsp
FJ978011.1	Pilot et al. (2010)	658		DNAsp
FJ978010.1	Pilot et al. (2010)	658		DNAsp
FJ978009.1	Pilot et al. (2010)	658		DNAsp
FJ978008.1	Pilot et al. (2010)	658		DNAsp
FJ978007.1	Pilot et al. (2010)	658		DNAsp
FJ978006.1	Pilot et al. (2010)	658		DNAsp
FJ978005.1	Pilot et al. (2010)	658		DNAsp
KF723526.1	Jansson et al. (2014)	431		DNAsp
KF723525.1	Jansson et al. (2014)	431	Wolf (NCBI)	DNAsp, MEGA, PhyML
KF723524.1	Jansson et al. (2014)	431		DNAsp
KF723523.1	Jansson et al. (2014)	431		DNAsp
KF723522.1	Jansson et al. (2014)	431		DNAsp
KF723521.1	Jansson et al. (2014)	431		DNAsp
KF723520.1	Jansson et al. (2014)	431		DNAsp
KF723519.1	Jansson et al. (2014)	431		DNAsp
Århus		242		MEGA, PhyML
Århus 2		245		MEGA, PhyML
Århus 3		253		DNAspMEGA, PhyML
Århus 5		223		DNAspMEGA, PhyML
Århus 6		219		MEGA, PhyML
Århus 7		218		DNAsp, MEGA, PhyML

Århus 8		218		DNAspMEGA, PhyML
Århus 9		241		DNAsp, MEGA, PhyML
Århus formodet		245		MEGA, PhyML
Danish Wolf	Unpublished	16.727		MEGA, PhyML
AF008158	Vila et al. (1997)	708	Coyote	MEGA, PhyML
AB007399	Tsuda et al. (1997)	673	Dog (NCBI)	MEGA