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Norwegian Lundehund – a model for genetic studies?

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Norwegian Lundehunds represent an extremely genetically homogenous breed. Accurate sex identification is not always possible by screening X-chromosomal loci, and additional molecular analyses are needed. Seventeen individuals were genotyped at 170 000 single nucleotide polymorphisms (SNPs) by high-density genome-wide SNP technology. Analyses with standard programs based on homozygosity of X-chromosomal loci failed in assigning individuals to the correct sex, which had been determined during sampling and reconfirmed with the sex-specific marker amelogenin. This demonstrates that theoretical sex estimations can be erroneous in highly inbred individuals. Secondly, we investigated the minimum numbers of male and female Lundehund ancestors using nine polymorphic canine Y-chromosomal markers and parts of the mitochondrial (mt) genome (1947 base pairs). In 57 Lundehunds, we identified only one Y-chromosomal and one mtDNA haplotype, which is consistent with only one male and one female being ancestor to the current population. This result also predicts extremely low genetic variation overall. Finally, all 20 examined Lundehunds were polydactylous and typed homozygous for the causative mutation identified for this monogenetically inherited trait in Western breeds. We propose that the Lundehund breed, due to its extremely low genetic variation, represents an excellent model for studying multifactorial diseases relevant also to humans.

Keywords

Norwegian Lundehund, ancestors, mtDNA, Y chromosome, polydactyly, sex identification

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3 41 **Introduction**
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5 42 Accurate sex identification is often uncertain or impossible for wild and free-ranging animals.
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7 43 Examples include samples with incomplete information such as non-invasive hair and faecal samples.
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9 44 Although poor quality data obtained by non-invasive sampling can result in low amplification success
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11 45 (Santini et al. 2007), information on individual sex from molecular markers provides important data
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13 46 for research and conservation (see *e.g.* Mumma et al. 2014). Sample identity problems can often be
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15 47 revealed by checking the reported sex of each individual against the one predicted by genetic data
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17 48 (Turner et al. 2011). Importantly, however, this requires reliable sex assignment. Despite genome-
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19 49 wide coverage from high-quality samples this may not be guaranteed.
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21 50 In the past, phylogenetic population studies in the domestic dog (*Canis lupus familiaris*) were mainly
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23 51 based on mitochondrial DNA (mtDNA) revealing information about their geographic and temporal
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25 52 origin (Savolainen et al. 2002; Boyko et al. 2009; Pang et al. 2009; Vonholdt et al. 2010) as well as
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27 53 their evolutionary history, such as *e.g.* backcrossing events with wild canids (Tsuda et al. 1997; Vilà
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29 54 et al. 2005). MtDNA, with its maternal pattern of inheritance, also provides information regarding
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31 55 maternal gene flow and phylogenetic relationships within and among purebred dog breeds. In
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33 56 addition, the paternally inherited Y chromosome allows conclusions about evolutionary events in
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35 57 paternal lineages of mammals. Thus, mtDNA and Y-chromosomal analyses yield information about
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37 58 sex-specific contributions at the time of the last bottleneck event after breed origin.
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39 59 The Norwegian Lundehund (FCI group 5, section 2, standard Nr. 265) is an endangered small Spitz
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41 60 breed that was developed to hunt puffins (*Fratercula arctica*) on steep cliffs in Northern Norway
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43 61 some centuries ago (Espelien 2012), and is characterized by unique breed-defining traits such as a
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45 62 great flexibility of joints and neck as well as the presence of extra toes in the fore and hind limbs
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47 63 (polydactyly; Park et al. 2008; Melis et al. 2013). These traits were probably under selective pressure
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49 64 as they may have given an advantage when hunting puffins (Galis et al. 2001) by preventing the dogs
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51 65 from slipping off the rocks. Preaxial polydactyly (PPD) is a common congenital anomaly of the limb
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53 66 with abnormal number of digits caused by alterations in the antero-posterior axis of limb development
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55 67 (for a review see Al-Qattan 2013). Human and murine PPDs are known to be associated with the
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57 68 same highly conserved gene *LMBR1* (limb development membrane protein 1 Clark et al. 2000) or
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more precisely with substitutions in an intronic regulatory element called zone of polarizing activity (ZPA) regulatory sequence (ZRS; Lettice et al. 2003). Canine homologous PPD has been mapped to CFA16 (Park et al. 2004), and it is caused by substitutions in a conserved intronic region of the *LMBR1* gene with limb-specific enhancer activity upstream of human ZRS (preZRS) as identified in Korean and Western dog breeds (Park et al. 2008).

The Lundehund has gone through at least two known severe genetic bottlenecks (Melis et al. 2013). The first bottleneck was caused by canine distemper in the 1940s and the second resulted from the abandonment of the little fishing village, Måstad (67°38′ N, 12°35′ E), which held the last remaining population in the 1960s. Today's population stems entirely from five surviving dogs, which shared a single grandmother. Three of them also shared the same mother (Frimann-Clausen and Laane 1968).

Our research had three separate objectives. At first, we use the endangered Lundehund breed, where male and female assignment was certified through independent observation and genetic analyses, to illustrate how theoretical assumptions in statistical genetics can produce erroneous sex estimates. This may be of concern for data analyses in general and conservation management of species at risk in particular. Furthermore, to estimate the minimum number of male and female ancestors in this dog breed, we investigate genetic relationships in a cohort of this breed based on both mtDNA and Y-chromosomal markers. Finally, to identify the cause of PPD in the Lundehund breed, we examine the known mutations in the conserved intronic sequence of *LMBR1* gene identified in Korean and Western dog breeds.

Materials and Methods

Two independent datasets were used, one for sex validation analyses and another for studying ancestry and PPD mutations. For sex validation, 52 Lundehund individuals were DNA-sampled by buccal swabs at an international breed meeting in Norway (2013). DNA was extracted using the Isohelix DDK-50 isolation kit. A subset of 17 individuals (five males and twelve females) were genotyped with the Canine Illumina High Density Beadchip (including more than 170 000 SNPs). Sex assignment was obtained using GENOME STUDIO V 2011.1 software (Illumina, San Diego, CA, USA) which estimates the sex of each sample using X-chromosomal SNPs. Similarly, the option "Sex

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check" in the software PLINK (Purcell et al. 2007) uses X chromosome data to determine sex (*i.e.* based on heterozygosity rates) and identifies individuals as males if the inbreeding coefficient F is higher than 0.8 and as females if F is lower than 0.2. Subsequently, PCR amplification with the amelogenin microsatellite marker for sex identification on all 52 samples was performed as described in Kekkonen *et al.* (2011), and alleles were scored using GENEMAPPER 4.0 software (Applied Biosystems). The percentage of missing alleles, observed (H_O) and expected heterozygosity (H_E) and relatedness (identity by descent, IBD) were calculated in PLINK based on 165 293 SNPs.)

For estimation of patri- and matrilineages, the study population comprising 57 Lundehunds (23 male and 34 female individuals) of different Norwegian stud book lines was characterized for Y-chromosomal markers and mtDNA. Blood and buccal swab samples were collected in cooperation with the Norwegian Lundehund Club. We isolated genomic DNA from peripheral blood cells according to standard protocols (Miller et al. 1988) and from epithelial mucosal cells with QIAamp DNA Mini kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. In order to estimate the number of male and female ancestors of the current Lundehund population we genotyped each individual at canine Y-chromosomal markers (five single nucleotide polymorphisms (SNPs) and four microsatellite loci) and sequenced a 1947bp sized portion of canine mitochondrial genome (NCBI accession number U96639) as described in a previous study (Kropatsch et al. 2011).

For PPD mutation analysis, we investigated a sub-set of male and female dogs (n = 20) of the aforementioned study cohort as well as four healthy Schapendoes dogs without PPD as controls. A 783bp-sized fragment of intron 5 of the *LMBR1* gene including preZRS with both PPD mutations (DC-1 and DC-2) for Korean and Western dog breeds, respectively (Park et al. 2008) was amplified using M13-tailed primers 5'-GCAAATGTATCACAGACATTGAC-3' and 5'-GATTGAGAAATAAGATCAATTTGATAAACA-3' and sequenced directly as described previously (Kropatsch et al. 2011) on an automated capillary DNA sequencer (ABI3500, Applied Biosystems, Darmstadt, Germany).

Results

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For sex validation, we obtained sex assignment by GENOME STUDIO that did not match that expected from sampling, *i.e.* all 17 individuals were identified as males whereas the amelogenin results were consistent with the sex assessed at sample collection for all individuals. For these 17 individuals, the average percentage of missing SNP genotypes was 0.71% and mean relatedness 0.899 (range 0.842 – 0.954). Across all SNPs H_O was 0.038 (SE 0.00031) and H_E was 0.035 (SE 0.00028). For the X chromosome (4982 SNPs), H_O was 0.036 (SE 0.00141) and H_E was 0.038 (SE 0.00149).

The analysis of the Y-chromosomal markers revealed that all nine markers were monomorphic in 16 of 23 males. For the remaining seven males, Y-chromosomal marker analyses failed because of poor DNA quality. We identified one Y-chromosomal haplotype or patrilineage (Table 1). In addition, one mtDNA haplotype or matrilineage (Table 2) was identified in all 57 individuals. Accordingly, stud book analyses revealed paternal gene flow from one known male ancestor called Kvikk 2 and one maternal lineage from one known female ancestor named Mosti (Figure 1).

PPD mutation sequence analyses revealed for the so-called DC-1 mutation of Korean dog breeds the wild type allele in homozygous state (G/G) for all investigated individuals including the controls, whereas the DC-2 mutation was identified in homozygous state (A/A) for all 20 Lundehund individuals. This was in contrast to all controls, which showed the wild type allele in homozygous state (G/G).

Discussion

As expected from high-density genome-wide SNP results with good coverage (> 99%), we identified in our Lundehund cohort almost the same low level of heterozygosity and high degree of inbreeding as found in previous studies based on autosomal microsatellite markers (Melis et al. 2013; Pfahler and Distl 2014). This extremely reduced level of genetic variation appears to result in male sex assignment for all dogs when using GENOME STUDIO or PLINK, despite 12 out of 17 being females. In current molecular genetic research approaches it is common to generate high-density genome-wide SNP data of the investigated individuals and simply infer each individual's sex based on these data. Thus in general, no further investigations are necessary. However, because sex estimates based on theoretical expectations in statistical genetics can be erroneous for highly inbred

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individuals and populations, it is important to consider the level of inbreeding when using programmes like GENOME STUDIO or PLINK. This is relevant especially in cases where sex identification is important for breeding, conservation or management purposes. In such cases, it may be necessary to perform independent tests *e.g.* amelogenin gene amplification which could be quite helpful also for poor quality DNA samples with low success in high density SNP genotyping.

Y-chromosomal and mtDNA data suggest that the current Lundehund population has one male and one female ancestor. Potentially, this observation only provides the minimum estimated number of male and female ancestors in the current population. It is for example possible that several Lundehund males with identical Y-chromosomal haplotypes contributed. In addition, we only investigated parts of the mt genome which could lead to an underestimation of the number of female founders as described (Pang et al. 2009). Nonetheless, our findings are consistent with the historical stud book analyses and the known breed history that the current global population originates from five dogs who were close relatives (Melis et al. 2013). Based on our results, we propose that perhaps only four autosomal, three X-chromosomal and one Y-chromosomal chromatids were provided by one male and one female ancestor, and hence represented in the Lundehund breed population.

Here, the extremely low genetic variation in the Lundehund was confirmed according to previous findings where autosomal microsatellite markers were employed (Melis et al. 2013; Pfahler and Distl 2014) despite the comparatively high mutation rates of autosomal microsatellite markers (Irion 2003). This implies also that non-synonymous mutations would be rare in this breed - at least in comparison to other breeds (see *e.g.* Cruz et al. 2008). Albeit variation in neither the Y chromosome nor the mtDNA have been identified within the study population, the age of the most recent mutations in the Lundehund breed can be estimated based on the number of generations since the last genetic bottleneck event. In addition, any hints for introgression of genetic variation from other breeds (see *e.g.* Fossum 1973) like Norwegian Buhund in the Lundehund breed were not evident from our analyses.

Genome wide association studies (GWAS) allow both monogenic traits such as susceptibility to Mendelian diseases and eye colour as well as various quantitative (polygenic) traits to be mapped using high density genomic data (Parker et al. 2004; Karlsson and Lindblad-Toh 2008). Dogs are

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especially suitable for such analyses because their demographic histories, with a bottleneck at the time of domestication from wolf (*Canis lupus*) combined with recent bottlenecks during breed creation, have resulted in long linkage disequilibrium (LD) within breeds (allowing for identifying regions at the Mb scale which contain genes of interest) and short LD across breeds (allowing for fine mapping of genes at the <100 kb scale; Wayne and Ostrander 2007; Karlsson and Lindblad-Toh 2008). With respect to the known breed history of the Lundehund, this breed shows the most extreme example of recent genetic bottleneck events and therefore has the lowest genetic variation ever measured in a dog breed (Melis et al. 2013; Pfahler and Distl 2014). Nonetheless, because of its genetic uniformity as a result of inbreeding and the small number of founders, the Lundehund is an excellent (semi-)natural model - independent from mouse and rat models - for studying polygenic disorders, which result from cumulative action of a number of different genes. The Lundehund breed is predisposed to a chronic form of gastrointestinal disease termed „Lundehund syndrome“ (LS) or „Lundehund gastroenteropathy“ which is multifactorial, meaning that several unknown genes may contribute to the manifestation together with *e.g.* even less well understood environmental influences. This disease is mainly characterized by clinical symptoms such as intermittent diarrhoea, vomiting, weight loss, lethargy, ascites, subcutaneous oedema of the hind legs and hypoproteinaemia (Landsverk and Gamlem 1984; Kolbjørnsen et al. 1994), but includes symptoms like gastritis, protein-losing enteropathy, intestinal lymphangiectasia and gastric tumours (Landsverk and Gamlem 1984; Qvigstad et al. 2008). It is assumed that LS is a wide-spread disease in the existing Lundehund population worldwide (Berghoff et al. 2007). Using highly inbred populations as a model for polygenic diseases provides the possibility to control for various environmental factors and allows one to focus on the few genetic differences seen between individuals, thus simplifying genetic analyses of polygenic diseases.

According to our results, the Lundehund breed is an exceptional animal model not only for polygenic but also for monogenic diseases such as polydactyly, which appears to be caused by the same intronic mutation in the preZRS of *LMBR1* gene as in the Western dog breeds Beagle, Cocker Spaniel, Malinois, Rottweiler, Shetland Sheepdog, Standard Poodle, Standard Schnauzer, Shih Tzu and Yorkshire Terrier (Park et al. 2008). Mutation sharing or rather sharing identical haplotypes in

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3 208 different breeds is commonly observed (Park et al. 2008; Kropatsch et al. 2011), yielding evidence for
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5 209 a common or shared origin (Bannasch et al. 2005), which is reflected in their breeding history (Sutter
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7 210 and Ostrander 2004). Although illustrating the common origin with other Western breeds, this
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9 211 mutation led to functional phalanges exclusively in the breed of Lundehunds. The serious health
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11 212 problems of the Lundehund might theoretically be in part the consequences of the pleiotropic effects
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13 213 of the mutation for polydactyly, which are affecting other parts of the limbs and the body (Galis et al.
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15 214 2001). Further research should clarify whether the health problems are instead independent
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17 215 consequences of the extremely high level of inbreeding.
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19 216 In recent years dogs have increasingly been used as a comparative model for human diseases and
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21 217 many of the findings from GWAS on dogs are now being translated to cohorts of human patients
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23 218 (Karlsson and Lindblad-Toh 2008; Slate et al. 2010; Tang et al. 2014). Therefore, studying the
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25 219 Lundehund syndrome might help understanding the interaction between genome and environment in
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27 220 similar autoimmune disorders caused by homozygous recessive mutations in humans and dogs, such
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29 221 as type I diabetes and inflammatory bowel diseases.
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43
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3 **Table legends**
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5 312 **Table 1** Single nucleotide polymorphisms (SNPs) and microsatellite markers on the Y chromosome
6
7 313 used for minimal male ancestor estimation and Y-chromosomal haplotype in Norwegian Lundehund
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9 314 individuals
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13 316 **Table 2** Identified mitochondrial DNA (mtDNA) haplotype based on variable sites in the mtDNA
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15 317 including the *ATP synthase subunit 6* gene and a 3'portion of *ATP synthase subunit 8* gene as well as a
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17 318 portion of D-loop sequence found in Norwegian Lundehund individuals.
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21 320 **Figure captions**
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23 321 **Figure 1** The ancestry of the Norwegian Lundehund breed based on stud book information. Squares
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25 322 and circles symbolize male and female dogs, respectively. Rhombuses correspond to descendants of
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27 323 unknown sex. Solid lines represent paternal thus Y-chromosomal lineages whereas dotted lines
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29 324 indicate maternal or mitochondrial DNA lineages. Question marks denote doubtful or unknown
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31 325 descent.
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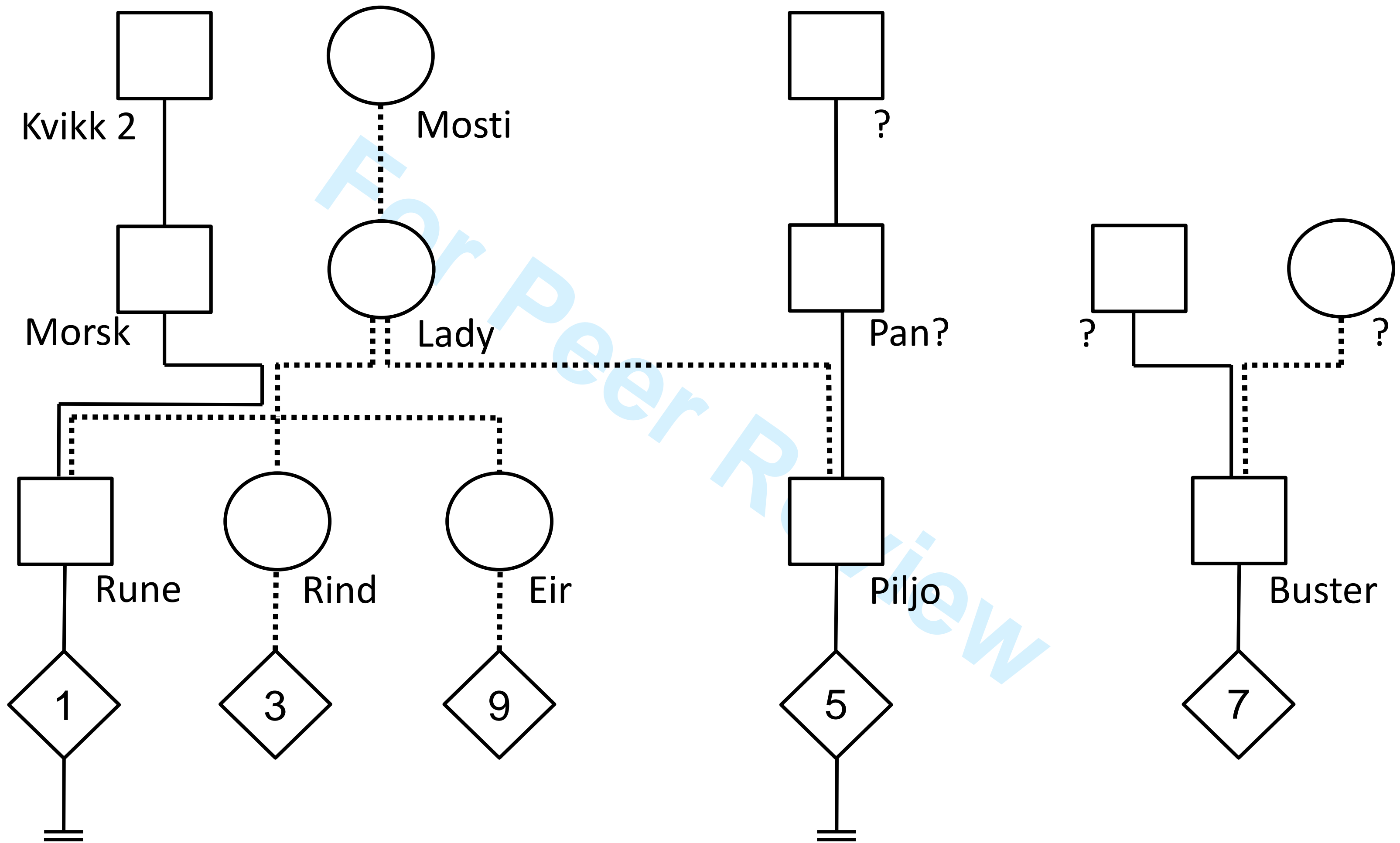
Figure 1



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Y chromosomal marker		Embl accession number	exchange position	(wildtype) allele	simple repeat motif	identified haplotype
SNPs	Ydog28	DQ973642	873	G	-	G
	Ydog21	DQ973639	417	G	-	G
	YdogG	DQ973680	219	T	-	T
	YdogN	DQ973627	608	C	-	GC
	Ydog20	DQ973692	35	A	-	A
microsatellites	MS41B	AF192268	-	224	$(CA)_4TAG(AC)_{19}$	224
	MS18	DQ973643	-	366	mixed repeat	370
	MS98	AY466398	-	151	$(CA)_{13}$	151
	MS97	AY466397	-	268	$(CA)_{14}$	268

For Peer Review

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mitochondrial DNA amplicons	exchange position	reference sequence U96639	identified haplotype
ATPase6 + ATPase8 genes	8368	C	T
D-loop sequence	15625	T	C
	15814	C	T

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