



KTH Biotechnology

Molecular Profiling of the Population Dynamics:
Foundation and Expansion of an Archaic Domesticated

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Abstract. An ‘exponential growth of science’ throughout modern history has been frequently boasted by numerous narcissistic accounts of ‘modern humanity.’ Nonetheless, ‘modern science’ seems to have overwhelmingly compromised on its promises, fitting into an ‘industrial scheme.’ With this concern, ‘molecular phylogeographics with conservational ambitions’ would look an intact ground for research efforts in a ‘school of biotechnology.’ The dog (*Canis familiaris*) as an earliest domestic animal has a history of conflicts over its origins and dispersal. Having those disputes addressed, valuable knowledge could be acquired on the nature and dynamics of domestication, and of human societies particularly of pre-agricultural ages. We employed two most widely-used genealogical markers, the mitochondrial DNA (mtDNA) and the non-recombining portion of the Y-chromosome (NRY), to address dog demography. Through 582 bps of mtDNA Control Region, complemented with whole mitochondrial genomes, it was established that almost all maternal lineages of the domestic dog worldwide coalesce to a population of at least 51 and perhaps many more female wolves in Asia South of Yangtze River (ASY) approximately 16,000 years before present (BP). This was based on the presence of a maximal diversity in this area, a descending gradient of diversity outward it, and a ubiquitous population structure everywhere in the world. A closer examination of this portrait in Southwest Asia (SwAsia) and the Fertile Crescent (FC), a region which has supplied persuasive evidence on early presence of the domestic dog, retrieved the same information, with implications for backbreeding with the local wolf population. Analyses of mtDNA dispersal showed that dogs took the long way via land to Madagascar Island, and not together with humans via sea. By the other approach, the NRY data in 14,437 bps length supplemented the mtDNA in reporting the height of diversity from ASY with a founding population of at least 13 male wolves, but expectably produced lower inter-regional differentiations by diversity. NRY screening of NRY by a SNP assay in the dingoes of Australia Island as a population of feral dogs revealed restricted and similar dispersal patterns for sires and dams. Prospects of ancient, multilocus and whole genome assays with the emerging high-throughput technologies has still more to promise on finer elaborations of these issues.

Keywords: Dog, wolf, dingo, mtDNA, NRY, SNP, Madagascar, Australia, domestication

There was a Door to which I found no Key:
There was a Veil past which I could not see:
 Some little Talk awhile of ME and THEE
There seemed—and then no more of THEE and ME.

Omar Xayyām Neyşāburi (1048–1131 AD)
English version by Edward FitzGerald

List of articles on which this thesis was based

Paper Zero.

Savolainen P, Zhang YP, Luo J, Lundeberg J, Leitner T (2002) Genetic evidence for an East Asian origin of domestic dogs. *Science* **298**, 1610–1613.

Paper I.

Pang J-F, Kluetsch C, Zou X-J, Zhang A-B, Luo L-Y, Angleby H, Ardalán A, Ekström C, Sköllermo A, Lundeberg J, Matsumura S, Leitner T, Zhang Y-P, Savolainen P (2009) mtDNA data indicates a single origin for dogs south of Yangtze river, less than 16,300 years ago, from numerous wolves. *Molecular Biology and Evolution* **26**, 2849–2864.

Paper II.

Ardalan A, Kluetsch CFC, Zhang A-B, Erdogan M, Uhlén M, Houshmand M, Tepeli C, Miraei Ashtiani SR, Savolainen P (2011) Comprehensive study of mtDNA among Southwest Asian dogs contradicts independent domestication of wolf, but implies dog-wolf hybridization. *Ecology and Evolution* **1**, 373–385.

Paper III.

Ding ZL, Oskarsson M, Ardalán A, Angleby H, Dahlgren LG, Tepeli C, Kirkness E, Savolainen P, Zhang Y-P (2011) Origins of domestic dog in Southern East Asia is supported by analysis of Y-chromosome DNA. *Heredity* **108**, 507–514.

Paper IV.

Ardalan A, Oskarsson M, Natanaelsson C, Wilton AN, Ahmadian A, Savolainen P (2012) Narrow genetic basis for the Australian dingo confirmed through analysis of paternal ancestry. *Genetica* DOI 10.1007/s10709-012-9658-5.

Paper V.

Oskarsson M, Ardalán A, Rabakonandrianina E, Savolainen P. African origin for Madagascan dogs revealed by mtDNA analysis. **Manuscript**.

Note: Tables S2 and S3 of Paper IV and Table S1 of Paper V are not included in this thesis due to the large sizes of the documents. The files are available upon request to the author.

CONTENTS

BACKGROUND 1

- Humanity and the significance of agriculture 1
- Mitochondrial DNA and its role in population studies 6
- Y-chromosome and its role in population studies 9
- The dog throughout history 11

PRESENT INVESTIGATION 15

- Sampling and general lab procedures 15
- History and motives 18
- On the origin of dogs 19
- On the dispersal of dogs 24
- On the admixture of dogs 30
- Debates and future perspectives 34

ACKNOWLEDGEMENT 37

REFERENCES 39

Paper I

Paper II

Paper III

Paper IV

Paper V

List of abbreviations used in this thesis

ASY: Asia South of Yangtze River

BP: Before Present

bp: base pair

FC: Fertile Crescent

FC-xtd: FC-extended

HG: Haplogroup

HT: Haplotype

ISEA: Island Southeast Asia

MYA: Million Years Ago

mtDNA: mitochondrial DNA

NGSD: New Guinea Singing Dog

NR1: Non-recombining portion of the Y-chromosome

PrASE: Protease-mediated Allele-specific Extension

SNP: Single-nucleotide Polymorphism

SEA: Southeast Asia

sub-HG: sub-Haplogroup

SwAsia: Southwest Asia

UT: Universal Type

UTd: UT-derived

BACKGROUND

Humanity and the significance of agriculture

Religion has remained the sole source of widely accepted and amenable knowledge on the origins of mankind, throughout history and prehistory, as well as in today's world. There have been early elaborations on the origins and development of life forms by Eastern scholars, among others largely original (Mattock 1971) descriptions by Jahez (776–868 AD), polymath from Basra (Cooperson 2008). He proposed a scheme of animal evolution that entailed natural selection, inheritance of acquired characteristics, and environmental determinism. He and also introduced the concept of food chains (Agutter& Wheatley 2008). However, it was only recently, by late nineteenth century, that these alternative views received, primarily in Europe, serious but minor consideration which continues to the present day. In his book of 1859 Charles Darwin annotated that the diverse forms of life have probably evolved from a common root through a branching pattern over the course of time, led by the force of natural selection (Darwin 1859). In a later edition Darwin draws “an historical sketch of the progress of opinion on the origin of species previously

to the publication of the first edition of this book,” tracing evolutionary ideas as far back as Aristotle (Darwin 1872). However, a limited awareness and/or appreciation of other nations, as is long rooted in West, likely prevented him from knowing about and/or mentioning several Eastern scholars who had proposed ideas similar to his own as far back as Early Middle Ages.

In a synthesis with the reintroduced Gregor Mendel’s laws of inheritance (Mendel 1866) which later on developed into what is known as quantitative genetics, Darwin’s theory eventually shaped the current evolutionary paradigm during the first half of the twentieth century (Bowler 1989). However, the foundation had to be still nurtured with numerous material and inferential evidence from linguistics, archaeology, paleontology, and later on from molecular genetics, in order to develop into a notable, though still minor, discourse on the mankind origins, as we know today.

Modern human belongs to genus *Homo*, which first appears as *Homo erectus* in fossils of *ca.* 2 million years ago (MYA) from Africa, and later on from other continents. This first human species supposedly developed from hominid ancestors which had furcated 5–7 MYA from the genus *Pan* encompassing our closest extant relatives, i.e. chimpanzee and bonobo (Jobling *et al.* 2004). The advent of the genus *Homo* is often marked by a significant increase in the brain size and by a widespread use of stone tools (Forster 2004). However, going by the latter the onset of *Homo* could be possibly extended to even 3.39 MYA (McPherron *et al.* 2010). The anatomically modern human (*Homo sapiens*) possessed cranial features such as increasingly globular skull and retracted face (Jobling *et al.* 2004). It probably developed *ca.* 200 KYA in East (Cann *et al.* 1987; Day 1969; McDougall *et al.* 2005) or possibly *ca.* 150 KYA in South (Tishkoff *et al.* 2009) of Africa, as marked by fossil and genetic evidence. Modern humans then spread out of Africa to inhabit Eurasia and Australasia, assumingly replacing the established populations of other hominids, and apparently interbreeding with some of them (Abi-Rached *et al.* 2011; Green *et al.* 2010; Reich *et al.* 2010). However, an alternative model assuming a

multiregional development of modern human throughout Afro-Eurasia has also been proposed in contrast with the Recent Single Origin Hypothesis, together with several intermediate models as well (Jobling *et al.* 2004).

The Anatomically Modern Human largely followed the lifestyle of his *Homo* ancestors in using stone tools and hunting and gathering mode of living, which involved dwelling in mobile bands lodging in temporary settlements based on proximity to water, edible plants and animal prey. By the end of Pleistocene geological epoch, all people on all continents had been living as exclusive hunter-gatherers (Diamond & Bellwood 2003). This was until the advent of agriculture at the start of Holocene epoch, which marks what is known as the Neolithic Revolution. The Neolithic or 'New Stone Age' as an archaeological period appears at different times in different regions of the world. A dramatic change in tool usage and particular cultural characteristics such as pottery distinguish this period, when access to food surplus led to the formation of permanent human settlements. This increasing sedentism gradually abolished the mobile hunting and gathering lifestyle. Instead, it encouraged trade and cooperation, and eventually yielded a 'complex society' organized in communities, states and empires during Bronze Age and later Iron Age (Jobling *et al.* 2004).

Thus, agriculture was a prerequisite for the development of the modern world as we know it. Notably, the shift to agriculture seems to be irreversible. It offers significant advantage over hunting and gathering in terms of accession to food supplies to support larger populations. Thus, for an ever-growing farming population it would be practically impossible to restore the previous lifestyle to earn a living based on hunting and gathering, by relying on the unchanged or even shrunk land resources of its habitat. Therefore, the number of the agriculturally-based populations may only increase over time. In fact, by now agriculture has enabled human to multiply since the end of Pleistocene his even-then-considerable population at 1000 folds (Jobling *et al.* 2004).

The farming innovation as the practice of improved food production was largely realized by the domestication of plants and animals for this purpose (Jobling et al. 2004). Production of food was based on initial domestication of relatively few wild plant and animal species in very few places. Most of these species, and the earliest ones, were plants (Crawford 2009; Zeder 2008), predominantly cereals and other grasses such as wheat, barley, millet, rice, corn, sorghum and sugarcane. The early animal domesticates were goat, sheep, pig, cattle and chicken (Diamond 2002). The domestic dog is probably an exception by being domesticated uniquely before the onset of agriculture, which makes it the earliest domesticate (Clutton-Brock 1995).

The early agricultural societies appear at different times in archaeological excavations from a small number of regions. The contents of these farming cultures also differ from one region to another, in terms of plant and animal species that were employed. Therefore, multiple and independent origins for agriculture may be inferred. However, the secondary centers of agriculture, which adopted this culture from elsewhere but added something to it, are not yet fully distinguishable from its original incubators (Jobling *et al.* 2004). The most important certain homelands of agriculture were Southwest Asia (SwAsia), Mesoamerica, and Southeast Asia (SEA). These regions were probably determined by the availability of species suitable for domestication and land resources to support farming (Diamond 1997). All these regions were of intermediary latitudes, the earliest likely being SwAsia with a date of 11,000 years before present (BP) for the birth of agriculture (Zeder 2008). This time corresponds to the Late Glacial Maximum when farming as an unusually successful cultural shift could spread rapidly over most of the globe (Jobling *et al.* 2004). The geographically uneven rise of farming innovation around the world reinforced the largest movements and replacements of human populations since the end of the Ice Ages (Diamond & Bellwood 2003). The spread of agriculture may take place in enhanced mode along an east-west geographical axes because of the climatic similarity among the areas at the same latitudes, demanding less adaptation by domesticates. Thus, farming culture

inhabited whole Eurasia in a more effective fashion, than did in the Americas and Africa, with a north-south geographical axes (Diamond 1997).

A ‘demic diffusion’ of the farming culture has been suggested, which involves an increasing population of early farmers who displaced or absorbed neighboring populations of hunter-gatherers (Ammerman& Cavalli-Sforza 1984). However, this model may be too simplistic. Alternatively, an ‘acculturation’ involving indigenous hunter-gatherers learning the farming techniques of their neighbors might have been partially responsible for the advancement of sedentary farming lifestyle (Jobling *et al.* 2004). Nonetheless, the idea of demic diffusion seems to gain more support by new genetic (Haak *et al.* 2010; Skoglund *et al.* 2012) and archaeological (Pinhasi *et al.* 2005; Pinhasi& von Cramon-Taubadel 2009) data.

Linguistic implications on the spread of agriculture arise from the fact that the representation of the world’s nearly 100 language families is very disproportionate in terms of their internal diversity and their geographic extents. Some language families are highly diverse encompassing many languages, and some are highly expanded inhabiting large parts of the world, and these two do not necessarily concord (Nichols 1997). The postulated homelands of some of the largest language families are within or near the first centers of agricultural innovations. This indicates that a link between the spread of agriculture and the spread of languages may have existed (Bellwood 2001; Renfrew 1987). However, there are known cases of languages which have spread irrelevant of agriculture, and that are not even spread in a ‘demic diffusion’ mode, i.e. accompanied with a significant gene flow. Instead, they seem to be ‘language shifts’ that are imposed by superior elites (Jobling *et al.* 2004), a phenomenon which could well fit into the term ‘acculturation.’ The dispersal pattern for populations and languages in connection with agriculture seems therefore to be very complex (Diamond& Bellwood 2003).

MtDNA and its role in population studies

The mitochondrion is an organelle of the eukaryotic cell, with varying numbers in each cell from zero to a few thousand, depending on the organism and the tissue. Mitochondria are the cellular sites of generating energy in the chemical form of adenosine triphosphate (ATP) through a process called cellular respiration. They produce 36 molecules of ATP in return of each molecule of glucose, which can demonstrate their critical role when compared with glycolysis, the cell's alternative mechanism of producing energy with 2 ATP molecules per glucose (Alberts 2008).

The mitochondrion as an intracellular structure was probably observed as early as 1841 (Henle 1841), in only a few years since the cell nucleus was discovered (Ernster& Schatz 1981). However, it was first established to be a ubiquitous organelle of the cell, then named "bioblast," in 1890 (Altmann 1890). Interestingly, the bioblasts were originally explained as "elementary organisms" that lived inside cells and had vital functions. This idea was revived in 1970 (Margulis 1970) when similarities of the mitochondrion to the structure of the bacteria had been noticed (Ernster& Schatz 1981). A free-living prokaryotic progenitor was suggested for these organelles, one that was bound with proto-eukaryotic cells about 1.5 billion years ago, in a symbiotic relationship involving energy generation by the prokaryote and being sheltered in a nutritious environment in return (Margulis 1970).

It became known in early 1960s, even before having arguably established an external bacterial origin for the mitochondrion, that this organelle has its own genome, functioning independently from the major genetic material of the cell in the nucleus (Andersson *et al.* 2003). The DNA of the mitochondrion or the mitochondrial DNA (mtDNA) was first discovered by researchers at Stockholm University (Nass& Nass 1963) and the University of Vienna (Schatz *et al.* 1964), using electron microscopy and biochemical assays, respectively.

The mitochondrial genome in fact resembles many features of the genome of the modern bacteria (Andersson *et al.* 2003). It is a double-stranded circular DNA molecule of 15,000–17,000 base pairs (bps) length in most mammals, and constitutes of a guanine-rich (heavy) and a cytosine-rich (light) strand. Each mitochondrion is estimated to contain 2–10 mtDNA copies , and a total of 100–10,000 copies of mtDNA are found in most cells (Alberts 2008; Wiesner *et al.* 1992).

The germ cells have a significantly lower mitochondrial content compared to somatic cells. However, it is commonly accepted that the inheritance of mtDNA occurs in a uniparental mode, and the egg cell alone gives rise to the total mitochondrial genome of an adult individual. This asymmetric contribution of the male and female lineages to the gene pool of the offspring is known as maternal inheritance (Alberts 2008; Giles *et al.* 1980). Thus, the mode of inheritance for mtDNA is non-Mendelian, since it is presumed in Mendelian inheritance that half the genetic material that makes up an individual is derived from either of the parents. There are reports also on the paternal inheritance of mtDNA (Zhao *et al.* 2004). However, evidence does not seem sufficient to have caused a shift in the paradigm (Jobling *et al.* 2004).

Inspired by the early studies on the origin of humans (Cann *et al.* 1987), a large number of all the studies of the origins of animal domestication have focused on mtDNA. The small circular mtDNA molecule has enough of conserved regions to allow universal PCR primers to work with the amplification of it. Nonetheless, it provides considerably high amount of diversity. Particularly, the hypervariable region of the molecule which is not coding proteins and is therefore almost neutral to selection is a site where an accumulation of mutations throughout the history of the molecule may be exposed in the form of different haplotypes (HTs). The mutation rate of mtDNA is five to ten times higher than the nuclear DNA in animals. This makes mtDNA a very useful molecule for the study of relationship and divergence between domesticates and their wild ancestors, due to the relatively short time since the start of

domestication, which is only 10,000 years (Zeder *et al.* 2006). Tracing the matrilineal descent of the domestic dogs to wolves is an example (Tsuda *et al.* 1997; Vilà *et al.* 1997).

One important feature of mtDNA in population studies is its mode of inheritance, considering the mating structure of the domesticated and many wild animals. The effective population size is much lower for male animals than for the female, and the progeny size for the reproducing males is considerably higher than that of the reproducing females. Also, particularly for the selective breeding practices in domesticates, there is a greater pressure on sires. They are easier to select and handle, and one would fertilize many females. Also, it has been believed since antiquity by many nations that the offspring are actually coming completely from the father, and that they only grow in size the mother's uterus. This could be found in the works of preformationist biologists of 17th century (Magner 2002). Accordingly, there is a tendency that breeders move the reproducing males in distances. Thus, unlike plants that are stationary, the past phylogeographical patterns for the paternal and biparental markers in animals are to some extent obscured, and inferences made of those possibly skewed. However, the maternally inherited mtDNA can be expected to show a higher degree of geographical correlation continued over time (Bradley 2000). On the other hand, some domestic animal species, particularly dogs, can interbreed with their wild relatives (Anderson *et al.* 2009; Klütsch *et al.* 2010; Vilà *et al.* 2005), and the resulting genetic introgression can make it difficult to reconstruct the phylogenies and history of the domestic population. However, this introgression should likely flow predominantly from the wild males mating with domestic females (Jobling *et al.* 2004; Vilà *et al.* 2005), as in the equally likely opposite case (Anderson *et al.* 2009; Iacolina *et al.* 2010) the offspring will be born to wild and will probably remain wild, thus not contributing to the domestic gene pool. Accordingly, mtDNA marker can advantageously exclude those introgressions from the wild.

For ancient DNA studies, mtDNA is more suitable than the nuclear DNA due to a high mutation rate giving sufficient differentiation from modern material, and the non-recombining mode of replication remarking a single lineage. The success of amplification of ancient DNA is also higher for mtDNA than for nuclear DNA (Jobling *et al.* 2004) considering its higher abundance in the organism, and possibly due to a higher chance of conservation in connection with size and shape of this small circular molecule. However, one complicating factor may be the problem of nuclear mtDNA insertions. The nuclear genome of human may contain over 400 kb of mtDNA sequences, which is 25 times as much as the mtDNA itself (Bensasson *et al.* 2001). Some of these insertions are very ancestral and some others are more recent to the individual carrying them. In case of modern DNA contamination, these insertions may be easily confused with ancient variants of mtDNA.

Y-chromosome and its role in population studies

The sex chromosomes X and Y possibly evolved from a normal pair of autosomes. Y-chromosome is the sex-determining factor in male mammals, and its inheritance is therefore exclusively paternal (Rice 1996). It has no homology with the X-chromosome over 90% of its 60 million bp length, and therefore they may not participate in meiotic synapsis and recombination as homologous autosomes do (Page *et al.* 2006). Recombination is particularly suppressed in the region containing the sex determination loci (Rice 1996), so the Y-chromosome can maintain its sex-determining function. This lacking or limited recombination prevents an exchange of fresh genetic material over the non-recombining portion of the Y-chromosome (NRY). Furthermore, it causes genetic inertness due to an absence of pre-recombination ‘mismatch repair’ mechanism that knocks out faulty DNA. While the X-chromosome still recombines with its homolog in female individuals, the Y-chromosome gets loaded with an accumulation of deleterious mutations and insertions of transposable elements. Much of the sex chromosome ancestral gene content is

estimated to have been lost from the Y-chromosome by now, and it has been suggested that this trend may lead to its degeneration or malfunction (Charlesworth & Charlesworth 2000). However, a comparative study shows that human Y-chromosome has conserved all its unique Y-linked genes since the time of divergence from chimpanzee, indicating more complex patterns in the dynamics of the Y-chromosome (Hughes *et al.* 2005)

Studies of the NRY have been conducted to reveal paternal history of the populations, which may not be necessarily concordant with their maternal one. This is due to different sociocultural niches occupied by the male and the female in connection with their roles in economy, marriage, war, etc. In one study (Seielstad *et al.* 1998), the observation on higher geographic correlation in NRY when in comparison with mtDNA was interpreted by ‘patrilocality,’ defined as a higher tendency for a wife to move into her husband’s natal household. This could be compatible with a patriarchal structure of the society. NRY studies are therefore highly informative in reconstructing demographic history and phylogenetic profile of the populations, although they may not provide as high resolution in terms of diversity as mtDNA can do. The mutation rate of the Y-chromosomal DNA is possibly the highest in the nuclear genome due to a mutagenic male germ line, although lower than mtDNA. Sequence divergence for the NRY is higher between species compared to that of other nuclear loci. However, its diversity is relatively low within a species, due to a small effective population size (Jobling *et al.* 2004).

A lower contribution of male animals to the genetic make up of the population is common in many mammal species due to the pattern and physiology of reproduction. In some livestock breeding agenda selective pressure is radically high for male animals. Even in a balanced population of males and females, there is one Y-chromosome for three X-chromosomes in the gene pool. With a considerably small effective population size, the Y-chromosome genetic material is highly prone to random drifts, which constantly removes diversity from the population, before it can take shape and stabilize. A mutation-drift

equilibrium can therefore not be reached as the enhanced drift outweighs the increased mutation rate. While this results in a very recent common ancestor and low diversity within species, the high genetic drift itself produces large differences between distinct populations, which makes the NRY a highly informative site with regard to geography.

The dog throughout history

The dog is supposedly the first domestic animal human ever owned (Clutton-Brock 1995). It was probably domesticated before the Neolithic (Dayan 1994; Raison 2005) and possibly remained the sole domestic animal for a few millennia. It is most commonly agreed that the origin of the dog was the grey wolf (Clutton-Brock 1995; Lindblad-Toh *et al.* 2005; Olsen 1985). However, other possibilities have been suggested, most prominently an origin from wild *Canis familiaris* “that voluntarily adopted the pariah niche and remained commensal for an extensive period before some populations became truly domesticated” (Koler-Matznick 2002). This would be compatible with the theory of ‘self-domestication’ (Crockford 2000)

It is not easy to distinguish remains of the domestic dog from wolf, and excavations from sites that are in connection with humans would therefore be most informative. The first evidence on the early domestication of canids are cranial alterations, including foreshortening of the muzzle, crowding of the tooth rows, and an overall reduction of the tooth size (Olsen 2000). The most distinctive cranial feature of a domestic dog is stated as the ‘turned-back’ apex of the coronoid process of the ascending ramus of the mandible. While this feature is frequently used as diagnostic for dog remains, it is also shared by the Chinese wolves and based on that the Chinese wolf has been suggested to be the wild ancestor of the domestic dog of Americas (Olsen & Olsen 1977). This could be further strengthened with the genetic evidence on an origin for the dogs of Americas in Afro-Eurasia (Leonard *et al.* 2002).

The oldest fossil that is conveniently assigned to domesticated dog is a part of a jaw which is estimated to be of 14,000 years of age (Street 2002), and was discovered in Germany. Also a cranial fragment and teeth from Kesslerloch Cave in Switzerland dated to 14,100–14,600 BP may represent an evidence of the domestic dog, as well as an early intermediary state between the domestic dog and wolf (Napierala& Uerpmann 2010). Another discovery of early domestic dog from Levant is estimated to be 12,000 years old (Dayan 1994). There are a few archaeological studies claiming dog remains earlier than the Late Glacial, such as remains of 13,000–17,000 BP from western Russia (Sablin& Khlopachev 2002) and of 11,500–15,000 BP From France (Pionnier-Capitan *et al.* 2011). Also, a dog-like canid skull was reported from an Upper Palaeolithic site (*ca.* 36,000 BP) in Belgium (Germonpré *et al.* 2009). However, the large size of some remains makes it difficult to confidently determine that they belong to domesticated dogs, and not to wolves that represent some cranial features of dogs. Another more recent study (Ovodov *et al.* 2011) claims dog remains from Siberia of 33,000 BP as an early domestication which was disrupted by the Last Glacial Maximum. A fresh study (Germonpré *et al.* 2012) also reports Palaeolithic “remains from hybrids or captive wolves.” There seems to be little fossil evidence for a transitional state between wolf and the smaller dogs, which leaves the question of the dog ancestry open. There are also a few genetic studies to suggest that the dog originated earlier than the Late Glacial Maximum (Lindblad-Toh *et al.* 2005; Vilà *et al.* 1997), in contrast with our findings. The site of dog domestication has also been subject to much debate, due to different and sometimes contradictory data from archaeological and genetic sources.

The reason to domesticate the dog is unclear and explanations vary from aid in hunting and protection to the consumption of the dog flesh. It has been most often suggested that the primary motivation for dog domestication may have been the role of their cooperation in the success of hunting. While this seems to be true (Ruusila& Pesonen 2004) it is hard to show whether it was the major

reason behind dog domestication. Moreover, this interpretation may be biased by a Eurocentric view which can not perceive dogs as food. The consumption of the dog flesh has been recorded in many parts of the ancient world (Titcomb& Pukui 1969; Wing 2000) as well as modern world (Olsen 2000; Simoons 1994), most of all from SEA (Higham *et al.* 1980; Simoons 1991). In his book of 1881 Bernhard Langkavel elaborates on how crucial food was in the survival struggle of early humans, and that they tamed the dog for its flesh (Langkavel 1881).

Dogs held an important stance in the cultural life of the earliest civilizations (Afshar 1990; Olsen 2000; Simoons 1994), as extensively recovered from the archaeological material (Clutton-Brock 1999; Olsen 1985; Przewdziecki *et al.* 2001). It has been suggested that proto-Indo-Iranians associated the dog with the realm of death (Afshar 1990), and there is evidence on ritual killing and burial of dogs for purposes other than eating (Olsen 2000; Wapnish& Hesse 1993). However, the Zoroastrians raised the dog to the position of symbolizing life and purity. As a guardian and sacred animal, it was considered a big offense if a person failed to feed his dogs, or mistreated or killed them (Simoons 1994). This notion is observed within other faiths influenced by Zoroastrianism, as restored in the iconography of Roman Mithraism, an early cult closely linked to Zoroastrianism and widely diffused among Roman legionaries (Smart 1996). It has been suggested that the impure picture of the dog in Islam may not be original to it, and has largely developed after the establishment of Islam in Iran, and in reaction to the unusually high regard of dogs in Zoroastrianism, the dominant faith until then (Afshar 1990; Goldziher 1901). Also, the importance of the impure picture of the dog in Judaism as the background to Islamic teachings has been stressed (Afshar 1990).

PRESENT INVESTIGATION

Sampling and general lab procedures

Sampling of canids from SwAsia as a region of interest to these studies was planned and performed by taking into account the requirements of project and the resources available to a then master's degree student of animal genetics. Samples were taken in the form of blood, hair and buccal specimens.

Hair sampling was convenient in terms of easy and non-invasive procedure. A major concern with hair sampling was whether or not the hair strands carried the root part, which contains enough of cells for DNA extraction. Experience showed that pulling hair from the animal's belly and inner thigh areas would increase the proportion of rooted hairs in the sample. Our work with mtDNA, which has a high copy number in the cell, helped the success of this sampling method. However, sufficient amount of DNA may not be achievable from hair samples for nuclear DNA work.

Buccal sampling using oral swabs and FTA cards was the common method we used through the process. This method worked very effectively, as it was totally non-invasive and required no expertise or equipments, and many people could help with sampling only by simple instructions provided to them. The samples on FTA cards could be sent by normal mail, and were possible to store at room temperature for long periods of time. Purification of DNA from FTA cards was relatively simple, and the quality of the DNA was very convenient, even by experiments with larger demands of DNA extract.

Blood sampling as an invasive method was relatively hard to be used on wild animals, as well as on their domestic relatives which were sometimes nothing short of wild. In fact, working dogs in rural areas can be very dangerous, due to lack of socialization periods and breeding for aggressive behavior toward intruders. There are other considerations regarding technical expertise and disease concerns, as well as transportation and storing of the blood samples, which make blood sampling not a method of choice. However, multiple blood samples were collected in vacuum tubes with EDTA anticoagulant, mostly in clinics. They were predominantly used to obtain sharp DNA extracts to provide reliable positive control for PCR experiments.

The dog samples were collected through sampling trips to different areas of the SwAsian region, with a particular preference for the sampling sites are located in rural areas rather than urban. This was due to the demography of the dog population throughout the region: indigenous animals, with distinct practical function under the local conditions, are most often kept in rural and suburban areas, while in urban areas people may tend to keep foreign breed animals, especially as pets, partially as a status symbol. These breeds were systematically avoided throughout the sampling process. Emphasis was given to sampling animals without known kinship, and in fact as separate as possible, both physically and by blood.

It was only with the help of many contributors that this work became possible. Some of these people were fellow students, and therefore already acquainted to working with animals. Sample collectors were provided with sampling instructions and in case of oral sampling, with FTA kits as well. The ‘foreign breed and kinship avoidance’ codes were explained to them, to be based on the looks and the owner’s account of the animal. In concern with rabies and also dog attacks not uncommon particularly in the suburban areas, warning was given to the collaborators, and they were asked to complete the job only if it was possible under safe conditions. In most cases they carried out sampling personally, and mailed the samples thereafter on their own charge.

Wolf sampling was performed in the form of hair samples at four wildlife parks, by collecting shed hair, as well as sampling directly from the animal’s body. No anesthetic procedures were permitted in order to get saliva or blood samples. One wolf bite accident happened, for which rabies vaccination was taken.

Red fox saliva samples were also taken at two wildlife parks for use in side projects. The animals were possible to handle manually with no anesthetic treatments due to the smaller size. However, another biting accident happened, which was managed with medical care.

Lab procedures started with treatment of samples to be prepared for DNA isolation, including enzymatic extraction from hair follicle cells and kit purification from buccal cells on FTA cards. The isolated DNA was taken to PCR amplification of the sites of interest, including mtDNA Control Region fragment, total mtDNA fragments, Y-specific fragment used for sexing of the samples, NRY single-nucleotide polymorphism (SNP) sites, and NRY fragments.

History and motives

In **Paper Zero**, which was a first comprehensive study of mtDNA in domestic dogs, it was established for the first time that the global dog population probably derived from one single origin in space and time, which was marked to be somewhere in East Asia at a time of around 15000 BP. This study included 654 dogs which represented all major dog populations of the world. The study concluded that more than 95% of all types of mtDNA in 582 bps of its length belonged to three major groups represented in certain proportions, and that this proportion evenly occurred throughout the world. This was interpreted as an indication for one single origin for all the three groups, since otherwise it would be unlikely for them to exhibit the same proportional frequencies everywhere in the world. In other words, if each of the three groups originated independently in one part of the world, there would be need for thorough mixing of the individuals affiliated with each of the groups with members of other groups at a world scale, in order for us to now come up the same proportions of the groups everywhere in the world.

Suggestion of East Asia as the center of origin for the domestic dogs was based on the finding that this region showed the highest diversity of HTs compared to other populations. Moreover, a gradient of diversity was detectable based on geographic proximity to East Asia, such that the closer region to East Asia showed higher genetic diversity. This left little doubt for the authors that SEA represents a center from which the dispersal of the dog population was initiated.

It was not possible for the authors to narrow down the geography of dog origination by the data they had in hand. A clear picture of changes in diversity was not either possible to draw. In an effort to provide detailed data on the origination of dogs, we moved to expand **Paper Zero** by setting elaborated analyses of the population structure and dynamics, and by extensive sampling

throughout the world, which would provide a more comprehensive representation of the worldwide dog population in **Paper I**.

On the origin of dogs

In **Paper I**, 1,543 dogs from around Afro-Eurasia were inspected for the same length of mtDNA addressed in **Paper Zero**. Also, 169 dogs were analyzed for their complete mitochondrial genome, in order to obtain higher phylogenetic resolutions. The adequacy of the samples surveyed in this study was verified by an independent study (Webb& Allard 2010) in comparison with several commonly cited evolutionary and forensic studies of dogs.

No new haplogroups (HGs) were found in addition to the ones described by **Paper Zero**. Nearly all dogs worldwide belonged to a common homogenous gene pool composed of three major HGs A, B and C, at similar proportions everywhere in the world, thus strongly indicating a single geographic origin for all dogs. This was due since with the three HGs having separate origins from different wolf populations, the locally obtained HG would have been carried by initially 100% of the dogs in each centre of origin, and would normally have remained at majority until today, unless extreme levels of population admixture, apparently unlikely, is assumed.

With the resolution provided by the whole mitochondrial genome analysis, each of the major HGs A, B, and C were found to be actually composed of a few sub-haplogroups (sub-HGs), summing up to totally ten HGs. As a measure of diversity, the full range of sub-HGs was found uniquely in SEA, in a region that was introduced as Asia South of Yangtze River (ASY), where the highest diversity for HTs was also present. The representation of sub-HGs decreased by distancing from this center of diversity with a gradient across Eurasia, so that in Central China seven of these HGs were present, to be followed by five in North China and SwAsia, and only four in Europe. The same pattern held

for HT diversity. The mean pairwise distance to ancestral HTs pointed to an origin 5,400–16,300 BP, when at least 51 female wolf founders were involved in the foundation of a primary dog population.

Paper II presented in this thesis was a second comprehensive study of mtDNA including additional samples from SwAsia, in reaction to a poor representation of this region in **Paper I**, and new studies remarking SwAsia as a possible origin of the domestic dog (Gray *et al.* 2010; vonHoldt *et al.* 2010), as well as questioning a Southeast Asian origin for it (Boyko *et al.* 2009). However, the methods used in these studies were disputable, as discussed in **Paper II**.

In challenge with our findings in **Paper I**, there are extensive archaeological elaborations on early presence of dogs in SwAsia, particularly in the Fertile Crescent (FC) area known as the heart of animal domestication (Davis& Valla 1978; Dayan 1994), as well as in Central Asia (Ovodov *et al.* 2011) and Europe (Germonpré *et al.* 2009; Napierala& Uerpmann 2010; Nobis 1979; Sablin& Khlopachev 2002; Street 2002). However, extensive archaeological excavations have not been carried out in SEA (Underhill 1997), and in one study it had been shown that the Chinese wolf is the ancestor of the American dog population (Olsen& Olsen 1977). In addition to that, there were concerns over an increased frequency of HG B and the presence of the local sub-HG d2, raised by **Paper I**. It was therefore of importance that a more comprehensive sample of SwAsian dogs should be analyzed, especially for the FC area, to ascertain whether a SwAsian origin for the dog could be established, whether HG B may have had a separate origin in SwAsia rather than together with HGs A and C in ASY, and whether sub-HG d2 is the result of a domestication of the wolf, or of dog-wolf crossbreeding.

Thus, **Paper II** analyzed 345 SwAsian dogs including 151 samples from the FC and its surrounding area, termed FC-extended, in the context of a universal sample set. Large samples from Persian Plateau and Anatolia were incorporated, mostly of working dogs of no specific breed from rural areas.

This was to address a criticism raised over **Paper Zero** underlining that since those authors “included many East Asian village dogs but few village dogs from other regions, their conclusion of high levels of East Asian diversity is likely a consequence of high levels of mitochondrial diversity in village dogs and not necessarily an indication of East Asian domestication” (Boyko *et al.* 2009).

The diversity for mtDNA among SwAsian dogs fitted into the pattern reported earlier for dogs across Eurasia in **Paper Zero** and **Paper I**, with a homogenous gene pool shared by all populations and carried by almost all individuals. No evidence of domestication in the form of unique HGs, high genetic diversity or strong deviation from the universal proportions of the major HGs was revealed in SwAsia. Also, within the FC-extended area the same HGs as in all parts of Afro-Eurasia were found, and almost all individuals carried a HT which was either shared with or differed by a single mutation from HTs that are spread across Afro-Eurasia, and except for those of sub-HG d2, could be traced back to HTs also found in ASY. The sub-HG d2, which was present at low frequencies in SwAsia represented an indication for a local crossbreeding with wolves. Other than that, no unique HGs or distinguished HTs were found in the FC-extended area, and the mtDNA diversity in there was significantly lower than in ASY, but comparable to other parts of Afro-Eurasia.

By the elaborations exposed in **Paper II**, it was once again established that ASY should remain the only region where virtually the full extent of genetic variation within the universal gene pool is represented. Thus a strong indication for ASY to represent the centre of origin for the world domestic dog population was provided.

As described earlier, the mtDNA data should be regarded as the most informative tool for addressing dynamics of the populations. However, in terms of origination adequacy of a maternally-inherited marker as the stand-alone reporter of the population foundation history may be disputable. Thus,

we set to obtain genetic data from NRY as a second independently inherited marker in **Paper III**, in order to assist the formulation of a scenario for the origins of the domestic dogs with improved evidence and thus better confidence. It would be unlikely that the supposedly independently inherited markers are affected by selection in the same way, or by chance take the same phylogeographical pattern. Therefore, similar results from NRY would highly strengthen our position on a Southeast Asian origin for the domestic dog. By this study, we would also obtain information not accessible via mtDNA, most importantly about the number of male founders and the extent of crossbreeding between female dogs and male wolves.

Based on an earlier study (Natanaelsson *et al.* 2006), **Paper III** was arranged as the first comprehensive study of NRY diversity among dogs worldwide. We analyzed 14,437 bp of NRY in 151 dogs, by which 28 HTs distributed in five HGs were defined. Two HGs showed a universal distribution, HG23 and H1. These HGs were present at relatively high frequencies and diversity in SwAsia and Europe, respectively, but were also moderately present in ASY. It was not possible to establish a geographical origin for these two HGs, and while it is not unlikely that they originated in their corresponding regions of maximal prevalence, a possible origin in ASY can not be ruled out for them. This is with regard to the higher rates of genetic drift in NRY, which commonly induces elevated levels of geographic correlations (Jobling *et al.* 2004). Three other HGs remained generally restricted to particular regions. Among them, HG9 was found at a very low frequency in two separate regions, Siberia and Central Africa. HG3 and HG6 were practically confined to eastern parts of Eurasia.

Like for mtDNA, nearly half of the total diversity for NRY was found at a worldwide distribution, while the other half occurred only locally. The highest extent of genetic diversity was found within ASY. Also it was possible to trace back all but one of the HGs to ASY, as it was only a single substitution, if not zero, that separated every HT in the universal gene pool from a HT which is found in ASY. This gives a virtually complete phylogenetic coverage for this

region. At least 13 wolf founders were inferred to have been involved in the foundation of the dog lineages studied in **Paper III**.

It could be concluded that the phylogeographic picture drawn by NRY matches that of mtDNA. **Paper III** provides indications that the highest diversity of NRY and virtually the full spectrum of it is only found in ASY, and there is now sufficient evidence at hand to recognize ASY as the major and possibly unique region of dog domestication. With considerable numbers of mtDNA and NRY wolf lineages found to be involved by both **Paper I** and **Paper III**, this domestication should have been conducted systematically at a large scale, and thus had extensive background in the cultural context where it occurred. It was suggested in **Paper I** that since the place and time of dog domestication roughly coincides with that of the domestic rice (*Oryza sativa*), the dog domestication might have been in connection with food production and not necessarily with hunting activities. This is concordant with the archaeological evidence on the dog flesh consumption tradition in SEA since early times (Higham *et al.* 1980) which has also survived into the modern day (Simoons 1991). It was also brainstormed that the domestication process may have had the substrate of scavenging wolves that approached human settlements, a theory known as ‘self-domestication’ (Crockford 2000).

The spread of the domesticated dogs from AYS over Afro-Eurasia seems to have taken place in a relatively efficient pace, in terms of fossil records and as discussed in **Paper I**. However, this process may not have worked so effectively for Island Southeast Asia (ISEA) and Australasia, where many ‘island hops’ were necessary to complete the spread. In our study of dingo origins in **Paper IV**, we tried to investigate the foundation events that led to the establishment of the dingo as a feral subpopulation of the domestic dog in ancient Australia. The aspects of these events are of ecological significance. It has been suggested that the introduction of dingoes into Australia Island led to the extinction of several species due to competition or direct predation. This is based on the evidence from zooarchaeological finds that suggest a coincidence

of these events (Archer 1974; Baird 1991; Corbett 1995). However, it has been also suggested that not necessarily dingoes, but actually humans may have had the major role in the extinction of Australian vertebrates (Johnson & Wroe 2003), and that dingoes may have actually had a positive effect of the conservation of the Australian biodiversity as a top predator (Johnson *et al.* 2007). A fine dating of the arrival of dingoes in Australia by genetic studies may resolve these debates. There have been efforts for the approximation of the arrival time by use of mtDNA marker (Oskarsson *et al.* 2011; Savolainen *et al.* 2004). However, more data from multiple markers need to be collected for this purpose.

On the dispersal of dogs

A number of HTs in **Paper I** were found to be present in all major areas of the world; these were termed Universal Types (UTs). It was also shown in **Paper II** that all populations across Afro-Eurasia are substantially UT-derived (UTd), which means that their individuals almost always carry a UT or a HT which is derived by one substitution from a UT. With a mutation rate of only one substitution per 40,000-155,000 years for mtDNA, a UTd may represent the very HT of the direct wolf ancestor. The ratio of UTd individuals is different among regions and varies by distance from ASY, ranging from 98% in North Continental Europe to 52.5% in ASY itself. Thus, ASY contains distinct HTs that never spread to other parts of the world to be once recognized as a UT. This would neatly depict a rich gene pool in ASY with an independent nature which gradually fades out along the axes of its dispersal, so that the genetic composition in remote areas may all in all be a reflection of this original diversity, with almost no indigenous resources of diversity to add to it. It was also shown in **Paper III** that except for one minor HG, all HTs of NRY worldwide are identical to or differed by a single substitution from a HT found in ASY, and may have potentially derived from such HT. The topology of the most parsimonious tree connecting the HTs followed a star-shaped pattern,

which would report on a population expansion (Jobling *et al.* 2004). However, a dispersal map based on sufficiently variable levels of intra-regional diversity was not possible to draw from the NRY data, and the rough commonality in the genetic contents among regions was only used as an indication for the commonality of the origin in the more inclusive gene pool. From the mtDNA data however, as discussed in **Paper Zero**, **Paper I** and **Paper II**, a clear picture of the spread was visible based on the gradient of diversity at large. Inter-regional similarities in the gene pools could therefore be used in more details to infer the actual routes of dispersal. In **Paper II**, closer ties of European dogs with SwAsia than with Siberia was indicated by a higher level of HT sharing between Europe and SwAsia. This suggested a prominently SwAsian route for the dispersal of the domestic dogs into Europe, possibly in connection with the Neolithic spread of agriculture.

Interestingly, the NRY data from **Paper III** revealed a distinguished diversity for FC region, mainly comparable only to that of ASY. Unlike that, the diversity of mtDNA in the FC region reported in **Paper II** was remarkably low. Also, the dog population in there deviated from normal proportions of the HGs, as was found in other parts of SwAsia and the rest of the world (roughly 60–85%, 10–30% and 5–15% for HGs A, B and C, respectively). Unusually high frequency of HG B, relatively low frequency of HG A, and lack of HGs C and D was typical of the FC-extended. Meanwhile, the same trend was observed in general SwAsian sighthounds, a morphologically distinct family which has evidently originated in SwAsia (American Kennel Club 2006; Przedziecki *et al.* 2001). This could be an indication for their specific origin in the FC-extended region. Interestingly, the same pattern was observed in Canaan dogs, a recently bred population which is genetically and morphologically very separate from the sighthounds, but is originating from the FC-extended (Levine 2003). This provides sufficient genetic evidence to pinpoint the dispersal source for sighthounds within the FC-extended region.

In an effort to collect more data on the male dispersal in **Paper IV**, we used the data from **Paper III** and an earlier study (Natanaelsson *et al.* 2006) to set up a Single Nucleotide Polymorphism (SNP) assay for screening Australian dingoes. The NRY length addressed in **Paper III** was landmarked by 30 polymorphic sites which defined NRY HTs among dogs. In **Paper IV** these diagnostic SNPs were used, together with one new SNP yielded from fresh NRY sequencing of two dingoes and one New Guinea Singing Dog (NGSD), performed to verify the dingo NRY as a feral dog type. With this SNP map, we designed an automated assay based on protease-mediated allele-specific extension (PrASE) technology (Hultin *et al.* 2005) to investigate the dispersal of dog paternal lineages into the Australia Island. Australia remained largely isolated after an initial inhabitation 50,000 BP from the New Guinea (Hudjashov *et al.* 2007; Ingman& Gyllensten 2003; Roberts-Thomson *et al.* 1996), and the dingo population in it was established by a dispersal of the domestic dogs from SEA less than 5000 BP, based on archaeological (Elledge *et al.* 2006; Smith& Litchfield 2009), ecological (Archer 1974; Baird 1991; Corbett 1995), and genetic (Oskarsson *et al.* 2011; Savolainen *et al.* 2004) evidence. It is known by studies of morphology (Corbett 1995) ethology (Smith& Litchfield 2010) and genetics (Savolainen *et al.* 2004) that the dingo is a feral subpopulation of the domestic dog. Dingoes are primarily wild, but they appear to have been in a semi-domestic relationship with humans and are important in native Australian culture as a representation of man in the animal world (Smith& Litchfield 2009).

A male-biased dispersal pattern in canids has been shown (Macdonald& Sillero-Zubiri 2004; Pal *et al.* 1998; Thomson *et al.* 1992), which could have caused higher rates of male contribution to an established population that remains isolated thereafter. With the otherwise largely isolated Australian fauna, assistance of humans in the spread of dingoes into Australia is most likely. If this was not true, a natural dispersal pattern would be possibly visible in a richer paternal than maternal ancestry.

Previous genetic studies indicated very low diversity for the Australian dingo population (Wilton *et al.* 1999). From studies of mtDNA it was known a single founder HT was present in Australia, and that all other HTs in Australia were unique, and had derived from it (Oskarsson *et al.* 2011; Savolainen *et al.* 2004). This implied that the dingo population was founded by a population of dogs which was very short in numbers or in diversity.

We screened 48 animals for NRY and found only two HTs present in Australia by a geographic pattern: H3 in the West, H60 in the Northeast, and both in Southeast. As shown in **Paper III**, the NRY H3 had been already identified in dogs, mainly from East Eurasia. However, H60 was specific to dingo and NGSD. It should be mentioned that although our sample was scarce, it was almost evenly scattered across Australia. In fact, it could be stated that the sample, although small, was a faithful representation of the diversity in Australia. The most densely populated region in Southeast Australia was effectively sampled, yet it reproduced the same proportions of the two HGs as the rest of Australia did.

The finding of the dingo-specific H60 in Northeast Australia and New Guinea by **Paper IV** was actually in agreement with other evidence. Beside archaeology, the full genetic diversity for Dog Leucocyte Antigen (DLA) in NGSDs was shared by the Australian dingo at 57% frequency on average of alleles (Runstadler *et al.* 2006), and the full genetic diversity for mtDNA in dingoes, going by the founder HT only, was shared by New Guinean dogs at 20% frequency (Oskarsson *et al.* 2011). The high degree of reciprocal sharing of the two gene pools in Australia and New Guinea indicates that dingoes were likely introduced from New Guinea.

It is therefore established from **Paper IV** and previous studies (Oskarsson *et al.* 2011; Savolainen *et al.* 2004) that the spread of dogs into Australia probably took place in a single introduction event from New Guinea, as indicated by a single mtDNA founder HT. Since there are several mtDNA HTs present in

today's Indonesia and New Guinea, multiple introductions of dogs into Australia should have brought different mtDNA founder HTs. However, this introduction event could still consist of more than one occasion, given that two Y-chr HTs are present in Australia. If a few dispersal waves were involved in the introduction event, they should have all originated from an already inbred population with very low genetic diversity. It would be also likely that the dispersal of H3 occurred at an earlier time, considering the farther spread of it into the West.

Nonetheless, genetic ties to a lesser extent with Bali Island has been shown through studies of microsatellite (Irion *et al.* 2005) and DLA (Runstadler *et al.* 2006), and therefore an additional dispersal route from Northwest Australia can not be ruled out. In such case, the central Australian desert between the two populations and a possible selective fitness for the arid climate gained by the Western group could have prevented an effective population admixture and thus yielded the NRY geographic structure. However, it seems not so likely that a gene flow from the West would also carry the sole founder mtDNA HT, given its low frequency in the Bali region (Oskarsson *et al.* 2011).

From **Paper IV** it is also inferred that the dingo male gene pool resembles the female one in terms of limited resources, as shown earlier (Savolainen *et al.* 2004). Thus it could be concluded that the dispersal of male and female lineages into Australia was probably mediated by humans via New Guinea, and not based on a natural male-biased pattern of dispersal. It seems clear that the Australian dingo population remained isolated after introduction, since any further admixtures would supply more diverse genetic material with 'heterotic' advantages that would stabilize it to be once visited by our studies.

An interesting place for the investigation of dispersal patterns is certainly the Island of Madagascar. Madagascar was one of the last major land masses in the world to be inhabited (Dewar & Wright 1993) by both humans and dogs. It has been shown that the first inhabitants arrived after 2000 BP (Burney *et al.* 2004;

MacPhee & Burney 1991) through migrations from ISEA, and that subsequent waves from Africa influenced the population structure of the island (Adelaar 2006). The Malagasy language belongs to the Austronesian language family, but other cultural elements are mainly African (Dewar 1995). Genetic evidence also shows that the Malagasy population forms an admixture of African and Asian gene pools (Hurles *et al.* 2005; Soodyall *et al.* 1995; Tofanelli *et al.* 2009). However, not much has been known as to whether the dog population on the island is connected with ISEA, Africa, or both.

In **Paper V** we analyzed 145 Madagascan dog samples for mtDNA Control Region, and compared with large samples from the all regions of the world, most importantly from potential parental populations in Austronesia and Africa, to determine levels of HT sharing between them. Strikingly, all Madagascan HTs were found to be attributable to Africa, either directly (90%) or by one mutational step (10%). However, by removing UTs, which are present everywhere and therefore not informative, the rate of sharing declined to 66%. Meanwhile, without UTs there was no sharing at all between Madagascar and Austronesia. We also extended comparisons to the regions surrounding the sea route for the migration of Austronesians towards Madagascar, mainly India and SwAsia. The results were 21% and 24% of sharing, respectively, by leaving the UTs out. This would anyway establish the Madagascan dog population as an admixed population.

In accordance with likely admixtures on the island of Madagascar, it was visible that this dog population contained much higher mtDNA diversity than an island population, for example Australian population, commonly does. It could be conceived that the dispersal into the island of Madagascar occurred at a massive scale, or alternatively multiple waves of gene flow affected the island, most likely from different departure points and thus bringing different material. With regard to the levels of HT sharing, the latter hypothesis seems to be more plausible.

It was concluded by **Paper V** that the dogs did not accompany humans on the long sea voyage, or did not complete it, and that their dispersal into Madagascar took place mainly by land. However, the spread over land proceeded very effectively, as inferred from the appearance of dogs in the fossil records from different parts of the world within a relatively short time span.

On the admixture of dogs

A study investigating origins of the dog in Americas published simultaneously with **Paper Zero** in the same magazine concluded that the ancient American dogs share ancestry with Eurasian dogs, and have an origin from Eurasian wolves (Leonard *et al.* 2002). The study finds that a diversity of dog lineages were brought to Americas 12,000 –14,000 BP, and thus concluded that the Eurasian dog population, possibly of multiple origins, should have been present as a well-mixed population during Palaeolithic, before their dispersal through Bering Straight into Americas. However, such extensive admixture seems to be unlikely given that trade was not effectively practiced among hunter-gatherer societies, and dispersal from a single origin which gave rise to all this diversity would be a more plausible scenario, as was shown in **Paper Zero**. A similar question in **Paper I** was addressed using population admixture simulations based on a simple stepping-stone model. It was concluded that very high migration rates would be necessary to give a degree of admixture that would produce the ubiquitous population structure that is observed everywhere in the world. In **Paper II** an extreme homogeneity in the dog populations across Afro-Eurasia was verified in terms of UTs: Outside ASY, 80% of the genetic content of all populations is composed of UTs, which are by definition shared by every population. This is a strong argument for a universal expansion from a single source population, unless such level of homogeneity was fundamental to the wolf population worldwide before domestication.

However, clear examples of indigenous genetic diversity are documented in a few regions of the world; the sub-HG d1 of Scandinavia addressed in an earlier study (Klüttsch *et al.* 2010), and the sub-HG d2 of SwAsia addressed in **Paper II** are two of them. The mtDNA HG D, to which the two sub-HGs d1 and d2 belong, has not been reported from East Asia, and therefore might represent a possibility for local independent domestication. However, as discussed in Klüttsch *et al.* (2010) and **Paper II**, the low diversity of d1 and the low frequency of d2, put together with a limited and detached geographic distribution of them implies a more likely formation through relatively recent local crossbreedings with female wolves. Also in **Paper III**, NRY HG9 represented one possibility for genetic introgression from male wolves. The HG9 was, as would be expected for a crossbreeding-derived HG, present only at very low frequencies, and also an outlier to the otherwise common gene pool across Afro-Eurasia. However, HG9 was found in far apart locations in Siberia and Central Africa. Thus, with a lack of regional occurrence for HG9, no indication of wolf crossbreeding could be postulated with this data. It is noteworthy that a male introgression into the domestic population should be more likely to happen and to be recorded than a female introgression. It could be conceived that with a higher resolution of NRY, a cleavage in HG9 based on geography should be possible to establish, as was due in the case of mtDNA HG D and sub-HGs d1 and d2 in Scandinavia and SwAsia. Also, with the relatively small sample size of the study, an indication of admixtures, if present, could have been missed. The mtDNA data had shown in **Paper I** and **Paper II** only minor effects of hybridizations in the composition of the dog gene pool. Our assessment from mtDNA and NRY data on wild ancestor genetic introgression into the domestic gene pool is inconsistent with the findings of a few previous studies (Vilà *et al.* 2005; Walker& Frison 1982), and arguably consistent with others (Vilà& Wayne 1999). We maintain however, by briefly concluding from **Paper I**, **Paper II** and **Paper III**, that almost all genetic diversity on both parental lineages, save for mtDNA HGs D and F and NRY HG9 may have been derived from a founding gene pool in ASY.

The NRY data from **Paper III**, once compared to mtDNA data from **Paper I** and **Paper II**, seems to follow a typical trend also observed in human population studies with NRY (Jobling *et al.* 2004): 1) lower diversity within populations and thus difficulty of differentiating populations in terms of their levels of internal diversity, which is likely due to smaller effective population size and the biochemical structure inhibiting mutation and recombination; 2) but meanwhile higher geographic specificity between populations which makes it possible to differentiate populations more effectively in terms of phylogeography, and is due to particular mating patterns such as ‘polygyny’ (male polygamy) and ‘patrilocality’ (male-oriented residence). Since patrilocality is defined only in human populations, and with actual evidence to its contrary on canid male-biased patterns of dispersal (Macdonald & Sillero-Zubiri 2004; Pal *et al.* 1998; Thomson *et al.* 1992) this concordance remains to be discussed, possibly by aspects of polygyny in dogs.

Similarly by the data from **Paper IV** it was visible that many more HTs of mtDNA were present in Australia Island as compared to NRY, which is likely due to a considerably higher mutation rate in mtDNA (Miyata *et al.* 1982) and a higher drift in NRY which has a lower effective population size. Nonetheless, the distributions of both markers were structured across Australia. The two NRY HTs, as well as the major mtDNA HTs, showed a gradient of distribution along east-west geographical axis (Oskarsson *et al.* 2011; Savolainen *et al.* 2004). However, the structured NRY distribution could be seen as more probably in connection with random genetic drift in a common gene pool, while the phylogeography of mtDNA may have been resulted from locally-evolved derivatives of the single founder HT A29 (Savolainen *et al.* 2004). Based on this, the cumulative evidence from NRY and mtDNA was found to be most compatible with a single founder event and a subsequent random drift within the established population.

By the study in **Paper IV** it was also meant to address the growing dingo conservation concerns with regard to an extreme genetic isolation of the dingo

as an archetypal dog. It has been discussed that outbreeding of the pure dingo population with modern dogs is a major threat to the future of the native dingo in Australia (Corbett 1995; Elledge *et al.* 2006). Compared to wolves that are behaviorally, physiologically and ecologically more distinct from dogs (Vilà & Wayne 1999), dingoes are closely connected with dogs, and interbreeding should likely occur more frequently. The hybrid offspring can gain higher selective advantage compared to the purebred resulted from crosses between inbred parents. This is due to an effect of ‘heterosis’ (hybrid vigor) which may evidently increase fitness by reversing the effects of ‘inbreeding depression’ (Shull 1948; van der Werf 2009). Thus, an effective population admixture seems to be overwhelmingly inevitable by the dingo being vastly exposed throughout Australia to modern dog breeds, being themselves highly inbred as well. Extreme genetic confinement of the native Australian dingo indicated by **Paper IV** and Savolainen *et al.* (2002), which in turn may enhance the height of heterotic effects in hybrids, implies that the serious conservationist concerns are indeed relevant.

The admixture addressed in **Paper IV** is due as a consequence to human population admixture in connection with European colonization of Australia. However, it was shown in **Paper V** that these processes may not necessarily always coincide. The dog population in Madagascar may be regarded as admixed, taking into account not only its extensive HT sharing with the African mainland, but also its lower extent of ties with SwAsia and India. However, this admixture does not accord with the human pattern of population admixture, which is largely influenced by spreads from ISEA. The complexity in the formation of population structures during the long course of time is partially exposed in **Paper V**.

Debates and future perspectives

The dog as an archaic domesticate holds a distinguished position throughout the history of humanity. It has aided human with hunting, herding, guarding, fighting, rescuing, detecting, worshipping, chores, pots, and of course company, at an era when humans hardly bear company of humans. Therefore, the origin and history of the dog should be of special anthropocentric interest to humans. Systematic efforts have been made via multiple disciplines, e.g. archaeology, paleontology, ethology, biology and the more recent molecular version of it to satisfy man's curiosity over the mysteries of dogs.

A direct comparison of the global dog and wolf populations may seem a straightforward approach to deduce the origins of dogs. However, available wolf data is restricted and the wolf has become extinct in large parts of the world, preventing a comprehensive comparison. As was shown in **Paper I**, the available phylogeny data show that the two principal dog mtDNA HGs cluster with wolf HTs: HG A contains HTs from North Chinese and Mongolian wolves, and HG B contains HTs from North Chinese, European, as well as SwAsian wolves. With regard to high levels of mobility in wolves, and that no universal picture on phylogeographic structure in wolves is available, this may not be found very informative.

Studies of molecular demography in populations may be easily affected by insufficient (Webb& Allard 2010) or biased sampling (Albrechtsen *et al.* 2010), as discussed in **Paper II**.

There have been disputes over the maternal inheritance of mtDNA, which may not be full (Zhao *et al.* 2004). Neutrality of mtDNA has also been subject to questioning (MacRae& Anderson 1988). Another issue which has been discussed, and that may affect demographic conclusions is the reliability of the data from online databases (James Harris 2003).

On the issue of dating past evolutionary events, legitimacy of using molecular clocks has been occasionally disputed (Bandelt 2007; Emerson 2007; Ho& Larson 2006). Also reaching higher levels of accuracy with radiocarbon dating is subject to ongoing research (Reimer *et al.* 2009).

More ambitions in the dog evolutionary research may include a better understanding of hybridization patterns among populations for the immediate purposes of conservation. The ongoing introgressions from wolf gene pool into the domestic and feral dog gene pools and in the opposite direction, extents and places of it should be of practical importance to the current canid research. Learning about the dynamics of dispersal and admixture in canid populations throughout history can reveal valuable facts about the history of human populations, too.

There has been arguments against certainty of the origination of the domestic dog from gray wolf (Koler-Matznick 2002) and the place and time of the domestication (Boyko *et al.* 2009; vonHoldt *et al.* 2010). Meanwhile, archaeological excavations in Southeast Asia have not been carried out in balance with other parts of the world, e.g. Europe and Southwest Asia (Bellwood 2005; Higham 1996; Underhill 1997) and not much is available in the literature on prehistoric dogs from mainland Southeast Asia (Higham *et al.* 1980). New archaeological efforts bringing in new data will change the scene for the dog domestication research. Also, methodology that enables us to conveniently distinguish ancient dog remains from other canids remains to be vastly improved. Built on that, the currently rare (Verginelli *et al.* 2005) ancient DNA work on canid remains may also get a boost. This may be facilitated by the emergence of the high-throughput analytical approaches offered by the new technology (Stoneking& Krause 2011).

Evolutionary study of populations based on only mtDNA and NRY may be insufficient to provide robust estimations of relevant population history parameters, as these markers address only two genetic systems, while evolutionary processes have a more inclusive stochastic nature (Destro-Bisol *et*

al. 2010). Multilocus analyses may have promises for addressing this difficulty (Tishkoff *et al.* 2009). In fact, what is known as domestication constitutes of multiple traits that are controlled by multiple loci and their epistatic effects. The ‘Quantitative Trait Loci’ (QTL) involved in the process of domestication can be more efficiently identified using the new high-throughput technologies on numerous genetic markers (Albert *et al.* 2009) or samples (Neiman *et al.* 2011). Large-scale assays or whole genomes may also facilitate the identification of the ‘major genes’ involved in domestication, which have been thus most affected by the domestication process. Having such a detailed picture of the genome available, it would become possible to address many questions on the history of animal and plant domestication, and thus of humanity.

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