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Department of Zoology, Institute of Ecology and Earth Sciences,
Faculty of Science and Technology, University of Tartu, Estonia

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Supervisor: Urmas Saarma, PhD, University of Tartu, Estonia

Opponent: Ettore Randi, PhD, Institute for Environmental Protection and Research (Istituto Superiore per la Protezione e la Ricerca Ambientale – ISPRA), Italy

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LIST OF ORIGINAL PUBLICATIONS

The thesis includes the following papers, which are referred in the text by their Roman numerals.

I. Saarma, U., Ho, S.Y.W., Pybus, O.G., Kaljuste, M., Tumanov, I.L., Kojola, I., Vorobiev, A.A., Markov, N.I., Saveljev, A.P., Valdmann, H., Lyapunova, E.A., Abramov, A.V., Männil, P., **Korsten*, M.,** Vulla, E., Pazetnov, S.V., Pazetnov, V.S., Putchkovskiy, S.V., Rõkov, A.M., 2007. Mitogenetic structure of brown bears (Ursus arctos L.) in northeastern Europe and a new time frame for the formation of European brown bear lineages. Molecular Ecology 16, 401–413.

II. **Korsten*, M.,** Ho, S.Y.W., Davison, J., Pähn, B., Vulla, E., Roht, M., Tumanov, I.L., Kojola, I., Andersone-Lilley, Z., Ozolins, J., Pilot, M., Mertzanis, Y., Giannakopoulos, A., Vorobiev, A.A., Markov, N.I., Saveljev, A.P., Lyapunova, E.A., Abramov, A.V., Männil, P., Valdmann, H., Pazetnov, S.V., Pazetnov, V.S., Rõkov, A.M., Saarma, U., 2009. Sudden expansion of a single brown bear maternal lineage across northern continental Eurasia after the last ice age: a general demographic model for mammals? Molecular Ecology 18, 1963–1979.

III. **Keis, M.,** Remm, J., Ho, S.Y.W., Davison, J., Tammeleht, E., Tumanov, I.L., Saveljev, A.P., Männil, P., Kojola, I., Abramov, A.V., Margus, T., Saarma, U. 2012. Complete mitochondrial genomes and a novel spatial genetic method reveal cryptic phylogeographic structure and migration patterns among brown bears in north-western Eurasia. Journal of Biogeography, doi:10.1111/jbi.12043.

* – maiden name

My personal contribution to the articles referred to in the thesis is as follows:

I – participation in material collection, laboratory procedures and article writing.

II and **III** – participation in material collection, laboratory procedures and data analyses; writing the first drafts of the papers.

1. INTRODUCTION

1.1. The study of phylogeography

Phylogeography is a discipline that focuses on principles and processes affecting the spatial distribution of genetic lineages, within and among closely related species (Avise *et al.* 1987; Avise 2000, 2009). Although the field is relatively new and the term *Phylogeography* has been in use for less than three decades, the number of studies using the approach has grown exponentially, with particular application to species in Europe and North America (Avise *et al.* 1987; Beheregaray 2008). Thus far, mammals are the group of organisms that has been most intensively studied using a phylogeographic approach (widely distributed species have been favoured) and, unsurprisingly, the main focus has been on human populations (Beheregaray 2008). Among wild mammals, the brown bear has become a model species, due to its wide distribution and the availability of numerous subfossil samples (Sommer & Benecke 2005).

Three genetic marker systems have been employed in phylogeographic studies of mammal populations: mitochondrial DNA (mtDNA), autosomal microsatellite markers, and sex chromosome markers, such as male-specific Y–chromosome microsatellites. Among these, mtDNA has been the preferred tool as it exhibits certain useful properties: 1) in most multicellular organisms it is usually inherited through the maternal germline without recombination, 2) it is present in cells in high copy number, and 3) it has a relatively high mutation rate, allowing the coalescence times between haplogroups to be estimated. In mammals, the mitochondrial genome is a double-stranded, circular molecule, including 13 protein-coding genes, 22 tRNA genes and two rRNA genes. In addition, there is a non-coding control region, which has a functional role in replication and transcription. Altogether, the length of the mtDNA molecule in mammals is in the range 15.3–17.7 kb (based on data of National Center for Biotechnology Information (NCBI)). The fact that mtDNA is usually maternally inherited makes it particularly useful for studying phylogeographic patterns in species like the brown bear where females tend to be more philopatric (i.e. they do not disperse far from their natal range) than males (McLellan & Hovey 2001; Støen *et al.* 2006). In such species, geographic structure in female-specific genetic markers tends to change more slowly and is therefore traceable during longer time periods compared to biparental and male-specific markers. As mtDNA occurs in cells in high copy number it is especially beneficial in studies of ancient DNA and it remains the key source of genetic information about late Pleistocene populations (Ho & Gilbert 2010). On the other hand, there are also potential pitfalls of using mtDNA: the molecule represents a single locus, evolution is not always neutral, and the mutation rate is not constant. Although maternally inherited, paternal leakage and recombination can sometimes occur (e.g. Galtier *et al.* 2009), suggesting that phylogeographic studies would benefit from using nuclear loci as well (Edwards & Bensch 2009). However, many of

these apparent problems with mtDNA occur at a low level (White *et al.* 2008), are of questionable importance in mammals (paternal leakage and recombination; Galtier *et al.* 2009) or can be accounted for analytically (e.g. the non-constant rate of the molecular clock can be modelled; see Ho *et al.* 2008b). The growing evidence that mtDNA deviates from neutrality (e.g. Elson *et al.* 2004; Ingman & Gyllensten 2007; Foote *et al.* 2010) is unsurprising considering that mtDNA is not functionally neutral as its genes are involved in the oxidative phosphorylation (OXPHOS) system. Despite this, Kivisild *et al.* (2006) found that a phylogenetic approach using mtDNA sequence data is adequate, as non-synonymous mutations mainly affect the terminal nodes of the tree and the reconstruction of internal nodes is robust. Considering all these circumstances, there is no reason to discard mtDNA from the phylogeographic analysis toolbox, especially considering the advantages of the mitochondrial genome as a matrilineal marker.

Typically, phylogeographic studies have used only a small fraction of the mitogenome (e.g. 2–10% of mtDNA in brown bears, typically the control region and/or a few protein-coding genes; see Davison *et al.* 2011), as the cost and effort required for sequencing more was previously prohibitive. However, it is widely known that an increase in sequence length can improve phylogenetic resolution. Advances in technology and reduction of costs have generated, albeit very slowly, an increasing number of studies where complete mitogenomes are used (the approach is far more widespread in human studies). Complete mitogenome data have been used for phylogeographic studies in the following non-human mammals: woolly mammoth (*Mammuthus primigenius*; Gilbert *et al.* 2008), cave bear (*Ursus spelaeus*; Stiller *et al.* 2009), dog (*Canis familiaris*; Pang *et al.* 2009), cattle (*Bos taurus*; Achilli *et al.* 2008), yak (*Bos grunniens*; Wang *et al.* 2010) and killer whale (*Orcinus orca*; Morin *et al.* 2010).

Mitochondrial phylogeographic data continue to be important in guiding conservation and management decisions, but more commonly aim to investigate the importance of historical processes acting on populations and species. Additionally, there is growing interest in studying functional processes associated with mtDNA and linking evolutionary biology with medicine (Galtier *et al.* 2009), as polymorphisms can be related to a number of metabolic and degenerative diseases, affect aging and permit intraspecific adaptations to occur (Wallace 2010).

1.2. Brown bear distribution, subdivision and phylogeography

Among eight extant bear species the brown bear has the most widespread historical and present-day distribution (Servheen *et al.* 1999). Historically, brown bears had a continuous distribution from Europe through Asia to North

America, and were also found in North Africa (Hall 1984; Servheen *et al.* 1999). However, during recent centuries bear populations have suffered from direct human persecution and habitat modification, especially in Western Europe, southern parts of North America, South Asia and North Africa, where brown bears have either disappeared entirely or decreased considerably, leaving behind small fragmented populations (Servheen *et al.* 1999; Zedrosser *et al.* 2001; Swenson *et al*. 2011). Currently, the largest continuous brown bear population is located in northern Eurasia, which includes half of the world population, estimated to exceed 200 000 individuals (McLellan *et al.* 2008).

As brown bears exhibit substantial morphological and ecological variability, hundreds of subspecies have been proposed worldwide. The early classification of brown bears is considered as a classic example of taxonomic over-splitting (Hall 1984). The most optimistic authors identified more than 90 subspecies in North America (Merriam 1918) and 271 subspecies in Eurasia (Ognev 1931), largely based on cranial and dental characteristics and often relying on examination of single or a handful of specimens. Later, the list of brown bear subspecies shortened substantially. Genetic studies, however, have revealed little or no concordance between mtDNA patterns and subspecies designations so far (Talbot & Shields 1996; Waits *et al.* 1998; Miller *et al.* 2006), and overall there is still no general consensus in brown bear taxonomy.

The first phylogeographic studies of brown bears were initiated by the need to identify potential conservation units in Western Europe for use in trans-locations towards small endangered populations (Randi *et al.* 1994; Taberlet & Bouvet 1994; Kohn *et al.* 1995). Since then, ancient and modern mtDNA sequences of brown bears have been extensively studied, and a number of clades and subclades identified (e.g. Talbot & Shields 1996; Masuda *et al.* 1998; Waits *et al.* 1998; Matsuhashi *et al.* 1999, 2001; Leonard *et al.* 2000; Barnes *et al.* 2002; Miller *et al.* 2006; Valdiosera *et al.* 2007; Zachos *et al.* 2007; Calvignac *et al.* 2008). Taberlet and Bouvet (1994) first identified two main mtDNA lineages in European brown bears, based on partial control region sequences: the eastern and western lineages. The Eastern lineage is composed primarily of large populations, whereas the Western lineage comprises small, fragmented and threatened populations. However, both lineages are distributed over large geographical areas, with a contact zone in Central Scandinavia, while in Romania these two lineages exist sympatrically (Taberlet and Bouvet 1994; Kohn *et al.* 1995; Zachos *et al.* 2008). Using more recent terminology introduced by Leonard *et al.* (2000) and extended by Miller *et al.* (2006) and Davison *et al*. (2011), the eastern lineage includes the following clades: subclade 3a in Eastern Europe, Russian Far East, Japan and Alaska; subclade 3b in Japan and Alaska/Canada; and clade 4 in Japan and Canada/USA. The Western lineage is present in Western Europe and Southern Scandinavia (clade 1), Alaska (subclade 2a) and in the circumpolar region (subclade 2b; polar bears (*Ursus maritimus*)). The phylogeographic positions of some clades (clades 5, 6 and Iranian bears) are still unclear, while populations in large parts of

continental Eurasia have received little attention due to the limited availability of samples (Miller *et al.* 2006). Additionally, analysis of ancient brown bear samples has revealed extinct clades and subclades (2c, 3c, North African) in Europe, North Africa and North America, suggesting that in the past the diversity of brown bears was even greater (Barnes *et al.* 2002; Valdiosera *et al.* 2007; Calvignac *et al.* 2008).

1.3. The roots of phylogeographic pattern

The Northern Hemisphere experienced dramatic climate and landscape changes during the Pleistocene (2.6 million to 11 500 years ago). During that period cyclic glaciations alternated with relatively short warm interglacials (Jouzel *et al.* 2007). Glacial expansions and contractions had a strong impact on the population size, distribution, genetic diversity and evolution of many temperate and arctic species (e.g. Stewart *et al.* 2010; Schmitt & Varga 2012). Populations increased their numbers and dispersed during suitable periods, whereas during unsuitable periods, numbers decreased and populations were restricted to refugia (Hewitt 2004; Kotlik *et al.* 2006). These processes left genetic footprints in populations and influenced their phylogeographic structure. Beside brown bear, a number of other mammals exhibit strong mtDNA-based phylogeographic patterns concordant with isolation occurring towards the end of Pleistocene *(e.g. Pteromys volans:* Oshida *et al.* 2005; *Meles meles:* Marmi *et al. 2006*; *Microtus agrestis*: Hermann & Searle 2011; *Mustela nivalis*: McDevitt *et al.* 2012). Strong phylogeographic pattern is especially well expressed in species with either low dispersal rates and/or maternal philopatry. By contrast, species with high dispersal ability and adaptability, like wolves (Vila *et al.* 1999; Pilot *et al.* 2010) and foxes (Teacher *et al.* 2011), often show little or no phylogeographic structuring due to erosion of any signal of population separation (Hofreiter *et al.* 2004). Alternatively, phylogeographic pattern may be minor or absent in species that were reduced considerably in numbers and survived only in a single refuge area; for example, pine martens (*Martes martes*) (Davison *et al.* 2001).

During recent decades, the general consensus has been that during glacial maxima, most temperate European species were largely restricted to unglaciated southern European peninsulas, only expanding back into mainland Europe once the glaciers had retreated (Taberlet & Bouvet 1994; Sommer & Benecke 2005; Hewitt 1996). Over the course of multiple glaciations, these refugial populations were believed to be repeatedly isolated to the same refuge areas, thus maintaining their genetic divergence (Hewitt 1996, 2000), whereas populations outside refugia became extinct (Stewart *et al.* 2010). Similarly, in North America, isolation into refugia in Beringia, Pacific coastal islands or southern mainland areas have been proposed to explain the maintenance of divergent clades (Talbot & Shields 1996; Barnes *et al.* 2002; Matheus *at al.* 2004). At the same time, large regions in northern Eurasia were probably ice-free during the

Last Glacial Maximum (LGM; approximately 22 000–17 000 BP (before present)), except areas in the north-west and north-east (including Kamchatka Peninsula) (Svendsen *et al.* 2004; Bigg *et al.* 2008) and the pattern of genetic differentiation in various species is rather different compared to Europe and North America. Many species in northern Eurasia show no substantial phylogeographic division, but clear signs of demographic expansion, based on contemporary mtDNA samples, indicating post-glacial expansion from one refugium (Fedorov *et al.* 2008). In Europe, there is increasing evidence that animal and tree species had wider distributions during the LGM than previously believed, and it is evident that many species also survived in so-called northern refugia (e.g. Stewart and Lister 2002; Kotlik *et al.* 2006; Valdiosera *et al.* 2007; Svenning *et al.* 2008; McDevitt *et al.* 2011; Schmitt & Varga 2012), as far north as Central-Scandinavia (*Picea abies*: Parducci *et al.* 2012). By analyzing subfossil samples from European mammals before the LGM, Hofreiter and colleagues (2004) failed to detect any phylogeographic pattern similar to those observed in extant species (incl. brown bear) and concluded that current phylogeographic patterns are transient relicts of the last glaciation and do not represent long-term adaptation to different environments. Similar conclusions were drawn based on brown bear samples found in North America (Leonard *et al.* 2000). It has also been proposed that fragmentation caused by human activities has played an important role in shaping current geographic segregation at least in some species, including the brown bear (Valdiosera *et al.* 2007).

Although there is large number of papers and species studied using a phylogeographic approach, there are still significant shortcomings in re-constructing the histories of plant and animal species, especially at large geographical scales. The location, size and the number of refuge areas, as well as the duration of isolation and the process of recolonization has been somewhat different between species, resulting in variations in phylogeographic patterns seen today (Stewart *et al.* 2010). Some common patterns exist, but it seems that each species reacted somewhat differently from others to the climate and landscape changes at the end of Pleistocene (Randi 2007; Stewart *et al.* 2010).

1.4. Divergence and molecular clocks

Estimating timescales for the divergence of different taxa is important when investigating speciation events and population subdivisions, linking the evolution of different taxa with paleoclimatic or biotic factors and formulating conservation priorities. Such estimates are often calculated using molecular clock approaches, which rely on appropriate calibration of observed sequence divergence. Usually one of three ways is used to incorporate calibration information into an analysis: (1) obtaining the age of a phylogenetic divergence event on the basis of independent fossil or biogeographic evidence (Weir & Schluter 2008), (2) importing a substitution rate obtained from independent data

(e.g. from other studies/species), or (3) including heterochronous sequences of known age (such as radiocarbon-dated DNA sequences) (Ho & Phillips 2009). However, considerable variation in molecular clock rates exists among genes, taxa and different DNA sequence sites (Ho *et al.* 2005). Additionally, the rate at which molecular clocks "tick" is not constant over time, and the nucleotide changes at external nodes (mutation rate) in phylogenetic trees can be an order of magnitude greater than at internal nodes (substitution rate) (Ho & Larson 2006; Ho *et al.* 2008b). As the mutation rate represents the nucleotide changes in segregating sites within species, some of which will be fixed, but most of which will be removed by drift or selection, the substitution rate represents only mutations that are fixed in the population (Ho *et al.* 2008b). The relationship between mutation and substitution rates (exponential decay curve) shows that molecular clock rate has a rapid decline between 0 and 1–2 My, indicating that this is the most challenging period for estimating divergence times and highlighting the importance of using a method that is able to distinguish the mutation rate from the substitution rate (Ho & Larson 2006). Unfortunately, this time window coincides with the species-population transition in many temperate species. For all of these reasons, the selection of an appropriate calibration approach has a crucial role to play in timescale estimations (Ho *et al.* 2007) and unsurprisingly there are significant discrepancies between timing estimates for the same divergence events depending on the method used (Ho *et al.* 2008b).

1.5. The objectives of this thesis

The main objective of this thesis was to examine brown bear matrilineal phylogeography in northern Eurasia, putative refuge areas during the LGM and postglacial migration processes after the LGM. Moreover, we aimed to investigate evolutionary relationships between bears from northern Eurasia with those in Japan and North America.

To achieve these aims, in paper **I** partial mtDNA sequences (388bp) were used to reconstruct the postglacial phylogeography of brown bears in eastern Europe and to calculate the formation times of different European brown bear lineages.

In paper **II** partial mtDNA sequences (1942bp) were used to analyse brown bear phylogeography across northern continental Eurasia and to reveal evolutionary relationships with lineages in adjacent areas, including Japan and North America. Bayesian divergence time estimates were calculated for different brown bear clades using a novel multiple-calibration approach.

In paper **III** complete mitochondrial genomes were sequenced to identify phylogeographic structure, signatures of demographic history and spatial processes for brown bears in north-western Eurasia. A novel, spatially explicit, individual-based approach was developed for identifying migration corridors and barriers.

The locations of possible refuge areas are discussed in all three papers.

2. MATERIAL AND METHODS

2.1. Sampling

Brown bear samples (mostly muscle, but also liver or hair) were collected during 1996–2007 from Estonia, Finland and Russia. All samples were from legally hunted animals (for purposes other than this study) or hair-trapped during fieldwork. Muscle and liver samples were stored in 96% ethanol at –20ºC. Hair samples were stored in paper envelopes prior the extraction of genomic DNA. Total genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN) or the High Pure PCR Template Preparation Kit (Roche Diagnostics), following manufacturers' protocols.

In publication **I**, 231 bear samples from Estonia, Finland and the European part of Russia (west of the Ural Mountains) were analysed, whereas 205 brown bear samples were used in publication **II** from a large territory in northern continental Eurasia, including the Asian part of Russia. In article **II** we also sequenced 20 western lineage bear samples from Romania (n=12), Greece (n=2), Italy (n=5) and Poland (n=1), and one polar bear sample (obtained from Tallinn Zoo; originating from Franz Josef Land). In publication **III**, 95 samples were analysed from Estonia, Finland and the European part of Russia. All these sample sets were largely overlapping.

2.2. Sequencing

Partial mtDNA was sequenced in papers **I** and **II**, and the whole genome was analysed in paper **III**.

In article **I** we sequenced 388bp of the mitochondrial genome, covering the 3' end of the cytochrome b sequence, two tRNAs and the 5' portion of the control region.

In article **II**, a 1952bp of mtDNA was sequenced: the complete cytochrome b sequence (1140bp), 5' and middle sections of the control region (663bp) and additional sequences (149bp) flanking cytochrome b and the control region. After removing the hypervariable pyrimidine tract due to its high level of homoplasy, 1942bp sequences were used for downstream analyses (note that the size is 1942bp rather than the 1943bp reported in article **II**, where a single monomorphic site was accidentally duplicated).

In article **III** complete mtDNA was sequenced using a newly developed set of 13 primer-pairs. Primer sequences and reaction conditions for PCR amplification, PCR product purification and sequencing can be found in the Materials and Methods of articles **I–III**. Sequences were resolved on ABI PRISM 377 or 3130xl automated DNA sequencers (Applied Biosystems).

In articles **I–II,** consensus sequences were created with the program Consed (Gordon *et al.* 1998) using sequence data from both DNA strands. In paper **III**

complete mtDNA sequences were assembled and visualized using Phred (Ewing *et al.* 1998), Phrap and Consed (Gordon *et al.* 1998) on a UNIX platform. Sequences were aligned with Clustal W (Thompson *et al.* 1994) in Bioedit 7.0.5.3 (Hall 1999) and corrected by hand when necessary.

2.3. Data sets

In article **I** two datasets were compiled: 1) 388bp sequences from 366 bears (including 135 samples from Saarma & Kojola 2007); 2) 206bp sequences from the 5' end of the control region from the same 366 bears plus 15 homologous mtDNA haplotypes (69 individuals) from GenBank to analyse the samples in a wider European context. The second dataset was also used for time estimations.

In article **II** two datasets were also compiled: 1) 1140bp (full cytochrome b) sequences from 205 bears sequenced in this study, plus an additional 145 homologous sequences available from Japan and North-America in GenBank, yielding a total of 350 sequences; 2) 1942bp sequences (full cytochrome b, the 5' and middle sections of the control region and additional sequences flanking the cytochrome b gene and control region) from 205 bears sequenced in this study. Time estimations were based on partial control region sequences of 196bp from our sequences and publicly available ancient and contemporary sequences from brown and cave bears.

In paper **III** analyses involved five datasets: 1) 257bp of 5' control region; 2) 1942bp (as in **II**); 3) nearly complete mtDNA (16686–16689bp; short segments in the control region were excluded from complete mtDNA due to their high level of homoplasy); 4) the complete protein-coding regions (11406bp) of the mitogenome, and 5) complete mitogenomes (16760–16793bp). In addition, the level of selection pressure was tested for 13 protein-coding genes.

2.4. Data analysis

2.4.1. Phylogenetic and demographic analysis

In all papers phylogenetic relationships between haplotypes were inferred using median-joining network analysis with the program Network (Bandelt *et al.* 1999) using the default settings.

Statistical tests implemented in DnaSP (**I** and **II**) (Rozas *et al.* 2003) or Arlequin 3.11 (**III**) (Excoffier *et al.* 2005) were used to test hypotheses of selective neutrality and to detect past population changes (Ramirez-Soriano *et al.* 2008). In article **I** Fu and Li's *F* (Fu & Li 1993) and Tajima *D* (Tajima 1989) statistics were calculated. Paper **II** additionally included calculation of Fu's *Fs* (Fu 1997) and Ramos-Onsins and Rozas R^2 (Ramos-Onsins & Rozas 2002), whereas paper **III** used only Fu's *Fs*. Three categories of neutrality tests are

classified on the basis of the information they use. Tests performed in our studies fall into two of these classes: Fu's *Fs* statistic belong to class II, which uses haplotype distribution data; the remaining statistics used in our analysis belong to class I and compare estimates of variable sites and mean pairwise differences between sequences (Ramos-Onsins & Rozas 2002). Class III statistics use information from mismatch distributions and have little power to detect population expansion (Ramos-Onsins & Rozas 2002). In these tests, deviations from neutrality can be a result of selective and/or demographic events, as negative values suggest a recent population expansion resembling positive selection (or, under certain circumstances, negative selection), whereas positive values suggest recent bottlenecks or purifying selection. As Ramirez-Soriano and colleagues (2008) found that the most powerful tests belong to class II and among these Fu's *Fs* is one of the most powerful, this statistic alone was calculated in paper **III**. Ramos-Onsins & Rozas (2002) examined the power of neutrality tests to detect population growth and also found that Fu's *Fs* was the most powerful, especially for larger sample sizes, whereas for small sample sizes R^2 should be preferred. Additionally, in conditions where the level of recombination is unknown, Tajima's *D* and R^2 are recommended (Ramirez-Soriano *et al.* 2008). Although not mentioned in paper **III**, we did not detect any signs of recombination in the NCmtDNA dataset using Recombination Detection Program software version 4.17 (Martin *et al.* 2010) and four recombination tests under the default settings: 1) Recombination detection program (RDP, Martin & Rybicki 2000), 2) Geneconv (Padidam *et al.* 1999), 3) Maxchi (Maynard Smith 1992), and 4) Chimaera (Posada & Crandall 2001).

DnaSP was also used to perform mismatch analysis in papers **I** and **II** and to analyse genetic polymorphism in paper **III**.

In paper **III** we also inferred a phylogenetic tree with Bayesian phylogenetic analysis implemented in the program MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). This analysis was performed to evaluate statistical support for mtDNA clades obtained with median-joining analysis. The HKY+G model of nucleotide substitution was determined as the best fitting model according to the Bayesian information criterion using jModelTest (Posada 2008) and a brown bear from the Russian Far East was used as an outgroup.

2.4.2. Identification of migration and potential movement barriers and corridors

In paper **III**, in order to investigate the strength and directions of brown bear migrations, Bayesian phylogeographic analysis according to Lemey *et al.* (2009) was performed on the protein-coding regions using program BEAST v1.6.1 (Drummond & Rambaut 2007). This probabilistic method has previously been used to reconstruct patterns of pathogen dispersal through gene flow, but it is generally applicable (Lemey *et al.* 2009). In this method, genetic information,

geographical locations and the age of each sample are used to infer the process of migration among discrete locations in timed coalescence phylogenies. A separate HKY model of nucleotide substitution was specified for each of the three codon positions and a discrete phylogeographic model was used, which allows rates of pairwise spatial diffusion to be estimated. Sampled bears were grouped into eight geographic categories, comprising two regions in Estonia, four in Russia and two in Finland. Forward and backward diffusion rates between each pair of locations were calculated. Significant diffusion rates were identified using Bayes factors, with a threshold value of 5.0.

A novel spatially-explicit individual-based approach was developed in **III** to identify regions or features in the study area that might represent corridors or barriers to migration. By interpolating the variance of pairwise genetic dissimilarity between individuals throughout the study area, we identified geographic regions where genetic dissimilarity between individuals was significantly higher or lower than expected at random i.e., representing possible migration barriers or corridors, respectively. For this analysis, a sample set of 82 bear individuals was used; those samples whose location was recorded with a precision more than 25 km were excluded. Based on the NCmtDNA dataset, a sample-wise matrix of the Jukes-Cantor index was calculated with MEGA 5 (Tamura *et al.* 2011). To remove the effect of isolation by distance (IBD), an asymptotic curve, modelling genetic dissimilarity in relation to geographic distance, was fitted through the points. Residual values from the fitted model (IBD residuals) were used as a measure of genetic dissimilarity with the effect of IBD accounted for. Using the locations of midpoints between sample pairs, we estimated the inverse distance-weighted average of sample-pair IBD residuals over a grid of points (step of 10 km) covering the entire study area. The resulting values of mean genetic dissimilarity at grid points were tested for significant deviation from a random pattern using 1000 permutations of the genetic dissimilarity data, while retaining the spatial layout of sample points.

2.4.3. Divergence time estimation

Bayesian estimates of divergence times and effective population size were calculated with BEAST. As mutation rate could not be directly estimated from the contemporary brown bear sequences due to the lack of a sound calibration point, calculations were based on radiocarbon-dated ancient brown bear sequences. The calibration strategy differed in publications **I–III**, depending on the available data and development of methods.

Intraspecific calibration was used in paper **I**, where the Bayesian analyses were performed to investigate the temporal framework of brown bear lineages in Europe. The mutation rates were estimated with program BEAST v1.3 from radiocarbon-dated sequences of brown and cave bears: from 35 radiocarbon-dated Beringian brown bear sequences obtained from ancient samples (Barnes *et al.* 2002, age range 9500–50 800 years). To compare the mutation rates

between brown and cave bears, 26 radiocarbon-dated subfossil cave bear sequences were used from Loreille *et al.* (2001), Hofreiter *et al.* (2002) and Orlando *et al.* (2002), ranging from 20 000–130 000 years. The time to the most recent common ancestor (TMRCA) of the European population was estimated, along with the TMRCAs of the Eastern and Western lineages. In addition to 29 haplotypes (435 individuals) from the Eastern lineage, 18 haplotypes (107 individuals) from the Western lineage were included from GenBank. All analyses were performed using the HKY model of nucleotide substitution (Hasegawa *et al.* 1985). Rate variation among sites was modelled using a gamma distribution with six rate categories. A coalescent model with constant population size was used to estimate the effective population size in each clade of brown bears.

In article **II**, instead of using only the intraspecific calibration, we decided to apply a multiple-calibration approach combining two distinct sources of information for age calibration: radiocarbon-dated ancient sequences (intra-specific calibration) and the age of cave bear-brown bear split based on fossil evidence (extraspecific calibration). The control region sequences from this study were combined with sequences from brown and cave bears that were publicly available from GenBank. The resulting alignment comprised 113 brown bears, including 47 ancient sequences (age range 400–50 800 years), and 25 ancient cave bears (age range 26 500–80 000 years). The age of the brown bear-cave bear divergence was calibrated with reference to the fossil record. A normal prior, with a mean of 1.45 Ma and standard deviation of 125 550 yr, was specified for this divergence event. Phylogenetic analyses were performed in BEAST v1.4.7 (Drummond & Rambaut 2007), using the HKY substitution model. Separate coalescent tree priors were specified for the brown and cave bear clades, each assuming a model of exponential growth (Ho *et al.* 2008a).

Divergence time estimation in paper **III** was limited to the analysis of third codon position sites from mitochondrial protein-coding genes to minimize the impact of purifying selection (Ho *et al.* 2005). The same prior as used in article **II** (with a mean of 1.45 Ma and standard deviation of 125 550 yr) was specified for the brown bear-cave bear divergence event. The resulting estimate for the age of the most recent common ancestor (MRCA) of the 95 sampled brown bears – with a mean of 26 580 yr and standard deviation of 5514 yr – was used to inform the lognormal prior distribution of the corresponding node in the Bayesian phylogeographic analysis. In order to obtain estimates of the MRCAs of clades of interest, monophyly was enforced on the five major haplogroups identified in both the network analysis and the MrBayes phylogenetic analysis described above.

2.4.4. Detecting positive selection

Two different analyses were performed to evaluate selective pressure among protein coding genes using the dataset from paper **III** (note that these results are not included in publication **III** and are presented here for the first time). All 13 mtDNA genes were analysed separately. DnaSP was used to calculate the ratio of the number of nonsynonymous substitutions (Ka) to the number on synonymous substitutions (Ks). The ratio of Ka/Ks (or ω) is widely used as an estimator of selective strength for DNA sequence evolution: $\omega > 1$ is indicative of positive selection, $\omega < 1$ of negative selection and ω close to 1 of neutral mutations. Positive selection was additionally tested with TreeSAAP (Woolley *et al.* 2003), which performs detailed analysis of selection on amino acid properties by categorizing the effect of physico-chemical changes that occur due to amino acid replacement into eight z-score categories and estimating whether amino acid changes deviate significantly from neutral expectations. Analyses were performed using aligned coding DNA sequences and the modified MM01 model (McClellan *et al.* 2005). The Bayesian phylogenetic tree was incorporated into the analysis and the genetic code was set to vertebrateMt; otherwise, the default parameters of TreeSAAP were used and sliding window size was set to 20 as recommended by the authors. All 31 amino acid properties were selected. Only the strongest values of selection were considered (categories 6–8 and significant ($p<0.001$) values over +3.09).

3. RESULTS

3.1. Phylogeographic structure of brown bears in north-western Eurasia

The matrilineal phylogeography of brown bears in north-western Eurasia (Estonia, Finland and the European part of Russia) was specifically analysed in papers **I** and **III**.

Based on a 388bp fragment of mtDNA (n=366) the brown bear population in Estonia, Finland and European part of Russia can be divided into three overlapping haplogroups, as revealed in paper **I** (Fig. 2). In all haplogroups most haplotypes, besides the central one, were rare, represented often by a single individual. The largest haplogroup (named as HG10 in **I**; 15 haplotypes; 62.9% of all individuals) was distributed throughout the whole territory, whereas the two others were identified only in Finland and Russia. Within the largest haplogroup, two haplotypes were characteristic only to Estonia, one of which was dominant and distributed throughout mainland Estonia. While the smallest haplogroup (HG7; 6 haplotypes, 10% of all individuals) was predominantly found in Russia, a single sample was also found in the north of Finland. Both haplotype and nucleotide diversity was highest in the third haplogroup (HG5; 11 haplotypes, 27.3% of all individuals), which was also widespread, but did not include brown bears from Estonia. Tajima's *D* and Fu & Li's *F* statistics were consistent with population expansion in the larger haplogroup and within the whole population (excluding the pyrimidine tract). For other haplogroups and for the whole population (including the pyrimidine tract) only Fu & Li's *F* was statistically significant.

By analyzing shorter mtDNA fragments (206bp) together with Eastern lineage sequences obtained from GenBank, a similar but less refined network was revealed (Fig. 3a in **I**). Some haplotypes that were previously distinct (based on longer sequences, 388bp) were no longer distinguishable. However, the GenBank Eastern lineage sequences were found in all three haplogroups. Highest diversity was found among Romanian samples, which were identified in all three haplogroups. The core of two haplogroups included bears from Slovakia and the core of one haplogroup included bears from northern Sweden and an ancient (~47 420 years old) sample from Austria. However, when the pyrimidine tract was excluded, only a single star-like haplogroup was obtained.

In paper **III**, five datasets comprising the same individuals (n=95), but different sections of mtDNA, were analysed to compare their power to resolve phylogeographic structure. Based on analysis of the shortest sequences (257bp and 1942bp; 6 and 18 haplotypes, respectively) star-like networks were obtained (Fig. 1a and 1b in **III**). Unlike in paper **I**, the pyrimidine tract was excluded from analysis in this paper. In common with the shortest sequence datasets (257bp and 1942bp), the central haplotype was numerous and widespread. However, when analysis was based on nearly complete mtDNA

(NCmtDNA; 37 haplotypes) five divergent, geographically confined and only partially overlapping haplogroups could be identified (defined as A, B, C, D and E; Fig. 2 in **III**). In addition, a network with the same topology was obtained based on the full protein-coding dataset, except that several branches were shorter and three haplotypes present in the NCmtDNA dataset were not evident here. Among these haplogroups, C was largely located in the east of the study area, whereas all other four haplogroups (A, B, D and E) were primarily located in the western part. The highest genetic diversity was observed in the eastern haplogroup (C), which dominates in the eastern part of European Russia and extends to Finland. Almost 90% of Estonian samples belonged to haplogroup B, which additionally included three samples from neighboring Russia and had the lowest haplotype diversity. The Finland-specific haplogroup (D) included three haplotypes distributed throughout Finland, comprising more than half of the Finnish samples. Haplogroup A was present in the western part of the study area, except Pskov oblast. The central (E) haplogroup was the least variable and located only in Russia.

In all haplogroups Fu's *Fs* statistic provided support for demographic expansion (Table 3 in **III**).

3.1.1. Migrations and potential movement barriers and corridors

Bayesian phylogeographic analysis of the NCmtDNA data supported six pairwise spatial diffusions out of a possible 56 (Fig. 3 in **III**). The most strongly supported diffusions, with Bayes factors (BF) > 10, were those from northern to southern Estonia ((BF > 173), eastern to northern Russia (BF > 109) and southern to northern Finland (BF > 81).

Spatially-explicit individual-based analysis of the NCmtDNA data indicated three areas where the interpolated mean Jukes-Cantor distance was lower than the value expected from isolation by distance alone (Fig. 4 in **III**). A possible migration corridor was detected in the area between western Vologda oblast, Leningrad oblast and Estonia, whereas in Pskov and south-eastern Finland high mean Jukes-Cantor distance indicated a possible migration barrier.

3.2. Wide-scale phylogeography of brown bears in northern Eurasia

Brown bears in northern continental Eurasia (n=205) formed two maternal haplogroups based on mtDNA cytochrome b (1140bp) sequences: Eurasian (E) and Kamchatkan (K) (Fig. 1 in **II**). The large and widespread Eurasian (E) haplogroup was very closely related to the smaller haplogroup (K) that was specific to Kamchatka Peninsula. These two haplogroups shared a close

evolutionary relationship with one of the haplogroups from Alaska (A) and with one from Japan (JA). The second haplogroup from Alaska (AL) and two others from Japan (JB and JC) were linked with Kamchatkan haplogroup (K). All these bears correspond to subclade 3a, using the terminology devised by Leonard *et al.* (2000). Most of the haplotypes identified in northern continental Eurasia (19 of 26) belonged to the very widespread haplogroup E (Fig. 1 in **II**). The central haplotype E1 was by far the most numerous, including 61% of analysed bears in northern continental Eurasia, and present throughout the area, even in Kamchatka. Another haplotype from haplogroup E found in Kamchatka was E3, which was not, however, detected outside this peninsula. Other haplotypes were represented by significantly smaller numbers of analysed individuals and had restricted geographical distributions. Only four haplotypes (E11, E13, E15 and E17) besides E1 were represented in more than one region in the European part of northern continental Eurasia. In haplogroup K the central haplotype K1 was the most abundant (53% of all bears in this haplogroup).

According to the 1942bp fragment of mtDNA (n=205) brown bears in northern continental Eurasia were divided into four closely related haplogroups: haplogroups EA and EB, which were widely distributed; and haplogroups KA and EC, which were specific to Kamchatka and Estonia, respectively (Fig. 3 in **II**). Most widespread and abundant was haplogroup EA (28 haplotypes of 50), which dominated to the west of the Ural Mountains, but was absent in Kamchatka. Haplogroup EB was less numerous (6% of samples), but nevertheless widespread and was found in Kamchatka.

When all haplotypes of both datasets (1140bp and 1942bp) were analysed using various population expansion statistics, such as Tajima's D, Fu & Li's F, Fu's F_S and Ramos-Onsins & Rozas R^2, they provided strong statistical support for demographic expansion in the Eurasian-Alaskan brown bear population at some point in the past (Table 3 in **II**). The same results were also obtained for smaller datasets and even for individual haplogroups (E and EA). Estimates of demographic parameters in the Bayesian analysis also revealed evidence of significant population growth. However, demographic expansion was not statistically significant in haplogroups other than E and EA, when they were analysed separately.

3.3. Divergence times of bear mtDNA lineages

Similar intraspecific substitution rates were obtained for Beringian brown bears and cave bears in **I**, 29.8% per million years (Myr) and 26.2% per Myr, respectively (which corresponds to 2.98×10^{-7} and 2.62×10^{-7} subs/site/year, respectively). The rate obtained from Beringian brown bears (mean 29.8%, standard deviation 10.8%) was used in further analyses of European brown bear sequences to estimate the time to the most recent common ancestor (TMRCA) and effective population size for Eastern and Western lineages and for the

whole European population. According to these calculations the most recent common ancestor (MRCA) of European brown bears lived around 175 000 years ago, while the European Western lineage was established approximately 70 000 years ago and the Eastern lineage 25 000 BP (Table 3 in **I**). Effective population sizes were 80 000, 45 000 and 30 000 individuals, respectively.

In paper **II**, the estimated substitution rate for brown bears was 1.97×10^{-7} subs/site/year. This rate was obtained using multiple calibration approach, where the mean mutation rates within terminal nodes of brown and cave bears (3.00×10^{-7} and 3.11×10^{-7} subs/site/year, respectively) was approximately six times higher than the rate along the basal branch connecting the two species. The TMRCA of various bear groups (worldwide, within brown and within cave bear lineages) were estimated. Using a combination of fossil and radiocarbon calibration, the TMRCA of brown and cave bears was estimated at 1.4 million years (Table 4 in **II**). However, the estimated age for the divergence was 266 000 years if the fossil calibration was excluded. Using a multiple calibration, the age of MRCA of all brown bears was estimated at 193 000 years. In a second analysis, performed only with radiocarbon calibration, a similar estimate was obtained. The median posterior TMRCA of Clade 1 (European Western lineage and extinct brown bears from North Africa) was estimated to be 56 000 – 81 000 BP according different estimations in **II** (Table 4 in **II**). Clade 3a was shown to have formed around 26 000 years ago (only brown bears analysed, using radiocarbon calibration).

Sequences of the mitogenome coding region from north-eastern European brown bears and the brown bear-cave bear divergence event were used for divergence time estimations in paper **III**. The mean substitution rate estimated for the whole coding region was 3.485×10^{-8} substitution/site/year. However, to minimize the impact of purifying selection, time estimates were calculated using the mutation rate of third codon positions, which was 7.543×10^{-8} substitution/site/year. The median posterior age obtained for all analysed brown bears in north-eastern Europe (in **III**) was 20.4 ka, while the median posterior ages of five individual haplogroups ranged from 7.7 – 15.2 ka BP (Table 4 in **III**).

Despite the fact, that divergence time estimations in all three papers had variations depending on the sample and sequence sets used and also in different calibrations of the molecular clock, the results are generally in good agreement (Table 4 in **III** and **Table 1**).

Table 1. Bayesian estimates of time since the most recent common ancestor (TMRCA) for mitochondrial clades of European brown bears (compiled from Table 3 in **I** and Table 4 in **III**)

Lineages		TMRCA (years ago)	
		Median	**95% HPD* interval**
European bears	Intraspecific calibration	174,400	60,790–313,800
European Western lineage		67,370	19,960–130,900
European Eastern lineage		24,420	5,790–49,500
North-eastern European bears (Eastern lineage)	Multiple calibration (interspecific + intraspecific)	20,429	12,206–29,737
Haplogroup A		15,179	6,820–24,527
Haplogroup B		8,538	2,504–16,888
Haplogroup C		11,154	5,077–19,447
Haplogroup D		7,737	2,934–14,875
Haplogroup E		9,346	3,454–18,081

* – 95% highest posterior density interval

3.4. General characteristics of brown bear mitogenomes and selection

The mitochondrial genomes of 95 brown bears from north-western Eurasia were sequenced. Sequence lengths varied from 16760 to 16793 bp, with length variation largely occurring in the control region (e.g. indels in the pyrimidine tract or length variation in tandem repeats). We also identified two indels in tRNA-Ser and in 12S rRNA (Table S4, **III**).

Nucleotide diversity varied considerably along the mitochondrial genome (**Fig. 1**). The most variable region was the control region, but after excluding homoplasious sections, it was no longer as diverse as several of the protein-coding genes (Table S4, **III**). The most variable genes were *ND4*, *ND4L* and *ND5*. *ND4* and *ND5* also had the highest average number of nucleotide differences per gene (data not presented). Genes with relatively low nucleotide diversity were *ND1*, *ND2*, *ND3* and *ATP8*. Among tRNAs the most variable were tRNA-Tyr and rRNA-Ser, while many tRNAs were completely conserved. To evaluate the relative contribution to the NCmtDNA network of each mitochondrial gene (and the control region), we expressed the number of mutations in a particular gene as a proportion of the total number of mutations in the entire NCmtDNA network and in particular haplogroups (A–E) (Table S5 in **III**). On this basis, the highest contributors to the NCmtDNA network were *ND5*, *ND4*, *CYTB* and the control region, and these same regions were also the highest contributors to individual haplogroups, though *COX3* also contributed

significantly to haplogroup A. However, when contribution was normalized in relation to gene length (Table S6 in **III**), a different set of regions exhibited highest contribution (tRNAs, *ND6* and *ND4L*).

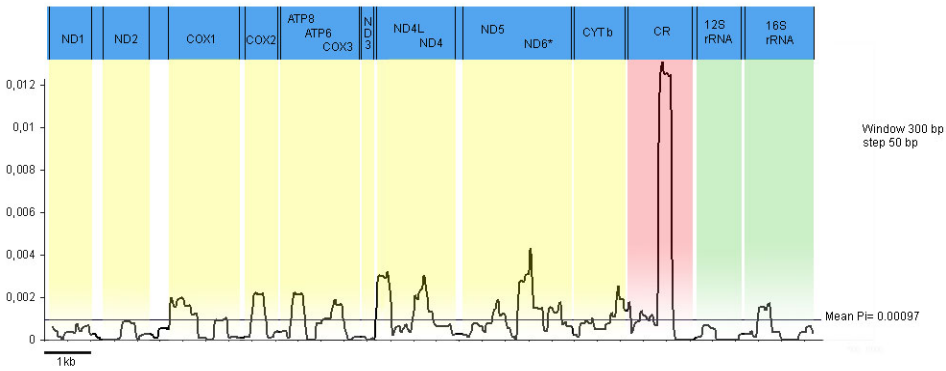

Figure 1. Nucleotide diversity along the complete mitochondrial genomes of 95 north-western Eurasian brown bears calculated using a sliding window method. Sites with alignment gaps are not counted in the window length. The boundaries of genes are approximate.

Note: white stripes represent tRNAs, the coding region is colored yellow, the control region red and rRNAs green. * – *ND6* gene is encoded on the L-strand of the mtDNA molecule.

In order to evaluate the level of selection acting on mitochondrial genes we used two different approaches. Positive selection was detected for the *ND6* gene, as the ratio of Ka/Ks was >1 (**Fig. 2**).

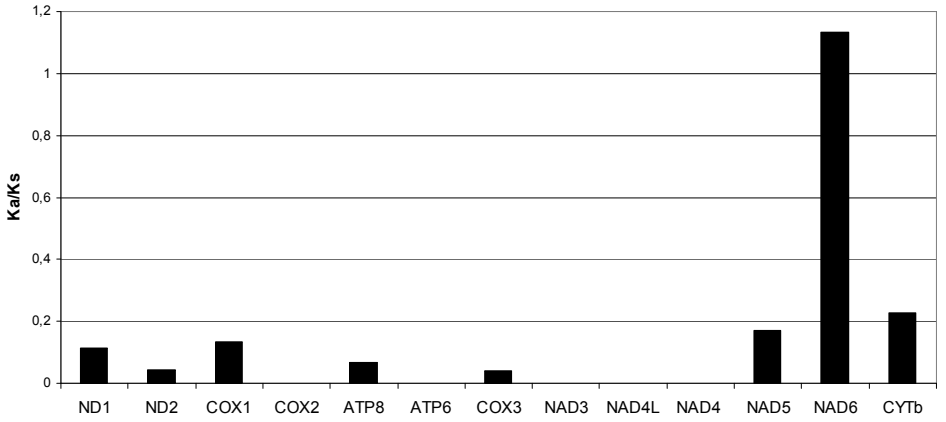

Figure 2. The ratio of Ka/Ks within different protein-coding genes in brown bear mtDNA (n=95).

Positive selection of *ND6* was also supported by analysis using TreeSAAP, where the z-score value of one property – power to be at the C-terminal – was positive and significant (category 6, value 5.173, $p < 0.001$). Out of 176 codons in *ND6*, we found seven mutations (one singleton and six parsimony-informative sites). Three of these mutations were synonymous: in codon 31 (Leu), 56 (Val) and 73 (Ala), whereas four mutations were non-synonymous: in codon 39 (Val↔Ala, mutation in codon position 2), 110 (Asp↔Gly↔Asn, mutations in positions 1 and 2) and 171 (Val↔Ile, mutation in position 1). In codons 39 and 171, non-synonymous substitution involved amino acids with the same polarity and charge, as valine, alanine and isoleucine are all neutral and non-polar. In codon 110, aspartic acid has negative polarity, whereas glycine and asparagine are neutral. Non-synonymous mutations in codon 110 are in the range where the z-score value of category 6 was significant.

4. DISCUSSION

4.1. Phylogeographic structure and migrations of brown bears in north-western Eurasia

All brown bears in north-western Eurasia belong to the monophyletic and relatively young subclade 3a. Based on sequences in **I** and **II**, star-like haplogroups emerged, suggesting a single origin and recent demographic expansion, as the central haplotype was present throughout the study area. Paper **II (**1942bp) showed that the same haplotypes may be recorded as far apart as Finland and Russian Far East, suggesting that bear population in north-western Eurasia is fairly homogenous and perhaps structured only by isolation by distance, and that recent common ancestry is the major factor determining the population genetic pattern. In contrast, using the nearly complete mitogenome sequences (NCmtDNA; **III**) we identified five haplogroups, with no haplotypes that were distributed throughout the north-western Eurasia. Instead, haplogroups were restricted to particular regions, exhibiting various degrees of overlap with one another, primarily in the western part of the study area. Hence, the sequences of nearly complete mtDNA as well as of the coding region offered significantly better phylogeographic resolution. Interestingly, the phylogeographic pattern of NCmtDNA is in relatively good accordance with genetic clusters derived from analysis of 17 microsatellites loci (Tammeleht *et al.* 2010), indicating that processes affecting male and female lineages could be rather similar.

Many brown bear populations in north-western Eurasia have suffered due to direct human persecution and habitat fragmentation. In general, the demographic history of bears has been more stable in the northern parts of the study area than in the western and southern parts (Pazhetnov 1993). Populations in Estonia, Finland and some oblasts of western Russia (including Tver oblast) experienced strong demographic bottlenecks during 19th and 20th centuries, when these populations were nearly extirpated, and from which they have at least partially recovered (Pazhetnov 1993; Valdmann *et al.* 2001; Saarma & Kojola 2007). Correspondingly, all haplogroups in north-western Eurasia were recorded to be undergoing demographic expansion.

The Estonian bear population came through the strongest known bottleneck at the beginning of the 20th century, when bears survived primarily in a large forested region called Alutaguse in north-eastern Estonia and the population was probably reduced to fewer than 30 animals (Kaal 1980). In the middle of the 20th century bear numbers started to increase, and during recent decades the population size has been relatively stable, with approximately 500–700 individuals estimated annually (Statistics Estonia). The signs of demographic history can be seen in the mitochondrial data, as the majority of Estonian bears carry a single haplotype, regardless of the sequence set (**I–III**). Although the bear population in north-western Eurasia is considered as continuous, the results

of mtDNA (**I–III**) and microsatellite (Tammeleht *et al.* 2010) analysis indicate that the Estonian bear population is genetically rather isolated. Based on 388bp (**I**) and 1942bp (**II**) sequences of mtDNA, the majority of Estonian bears carried haplotypes unique to Estonian population, and analysis with nearly complete mitogenomes revealed a mainly Estonian-based haplogroup (B), in which only a few individuals were also found in adjacent areas of neighboring Russia (**III**). The latter may indicate either migration from Estonia to Russia, or a historically wider distribution area of this particular haplogroup. However, it seems highly plausible that water bodies (e.g. Lake Peipsi, Narva River and Narva Reservoir) reduce the migration between Estonia and Russia, while the Gulf of Finland is a barrier between Finnish and Estonian bear populations. Results using biparental markers indicate that infrequent gene flow occurs between Estonia and Russia, probably in both directions (Tammeleht *et al.* 2010). The spatially explicit analysis in **III** indicates that a putative migration corridor is situated between north-west Vologda oblast and Estonia, which coincides with the southern border of the taiga forest biome (after Olson *et al.* 2001). The Bayesian analysis also detected moderate migration from South Estonia to West Russia, which probably represents the same area. Differences between the results of spatially explicit and Bayesian analysis may result from the fact that the former uses an individual-based approach while the latter grouped individuals into populations according their locations. However, within Estonia a strong migration trend in a north-south direction was detected with the Bayesian analysis (**III**). The core-area of Estonian bears is still in north-eastern Estonia and it has been shown that in expanding populations females disperse more efficiently, though still not as far as males (Swenson *et al.* 1998; Kojola & Laitala 2000).

The spatially explicit analysis detected a migration barrier in Pskov oblast, restricting migration between Southern Estonia and adjacent regions in Central Russia. This outcome is supported by the fact that bears do not inhabit the deforested areas in the centre of Pskov oblast (Danilov *et al.* 1993). However, additional sampling in both areas is needed to confirm this prediction.

Similarly to Estonia, bear numbers in Finland were lowest at the beginning of the 20th century, when the bear population was restricted to the northern and eastern parts of the country (Saarma & Kojola 2007). However, the current bear population in Finland is surprisingly heterogeneous. Based on nearly complete mtDNA three haplogroups were detected and both haplotype and nucleotide diversity was relatively high (**III**). Based on shorter sequences, the Finnish bear population is also quite diverse (**I** and **II**), though based on 388bp (**I**) more than 60% of bears in Finland carried a single haplotype. This heterogeneity perhaps reflects the observation that the recovery of this population was supported by migration from neighboring Russian Karelia (Pulliainen 1997; Kojola *et al.* 2003), and according to 14 microsatellite markers, bears in Eastern Finland and Russian Karelia also group into a single cluster (Kopatz *et al.* 2012). Most bears in Finland live along the border with Russia; however, during the last decade the population in Southern Finland has been expanding (Saarma & Kojola

2007). Population density in northern Finland remains low, mainly due to reindeer-herding practices, which are accompanied by special legislation to remove large predators (Kopatz *et al.* 2012). However, Bayesian analysis demonstrating a migration trend from southern to northern Finland (**III**), most probably reflects a wider historic movement pattern and not so much a recent population expansion at local scales, as one decade is too short a period to leave any detectable genetic signal even at the level of the whole mtDNA.

Almost 75% of European brown bears inhabit European Russia (Zedrosser *et al.* 2001). Based on the results derived from NCmtDNA sequences, four haplogroups out of five were identified in European Russia, indicating a complex population history after the LGM in this area. As both maternal haplogroups and biparental genetic clusters (Tammeleht *et al.* 2010) are to some extent overlapping between European Russia and Finland, populations in these two countries can be considered as continuous.

The results for Eastern lineage brown bears in paper **I** allow hypotheses to be generated about the locations of possible glacial refuge areas, as well as directions of re-colonization after the LGM. In light of these results (**I**) the most appropriate candidate for the refuge area was the Carpathian mountain range, also supported by the presence of brown bear subfossil records from this area (Sommer & Benecke 2005). The Carpathian Mountains has been proposed as a refuge area for many other temperate species during the LGM (e.g. Jaarola & Searle 2002; Kotlik *et al.* 2006; Ursenbacher *et al.* 2006; McDevitt *et al.* 2012; Schmitt & Varga 2012). Considering the whole of northern continental Eurasia (**II**) it seems that after the LGM bear populations radiated across the large part of northern continental Eurasia from a refuge area somewhere in Asia.

However, it is not actually clear whether the ancestral population was limited to a single LGM refuge area or whether it was presented in larger area or multiple areas, as indicated by the pre-LGM timing of the MRCA and LGM-dated fossils from sites in Carpathian and Ural mountains, and further east in Russia (Davison *et al.* 2011). Nevertheless, this does not mean that there were no additional refuge areas during the LGM, as not all refugial populations necessarily contributed to contemporary populations (Herman & Searle 2011; Stewart 2012). In light of all our results, it seems plausible that the eastern lineage bears migrated after the last ice age to northern Europe from two refuge areas: from the Carpathian Mountain range in the south, and from a refuge in the central or eastern Eurasia. However, establishing the number and exact locations for refuge areas is difficult, requiring intensive sampling and in addition also genetic analysis based on ancient DNA samples.

4.2. Phylogeography of brown bears in northern Eurasia and their evolutionary relationship with bears from adjacent regions

Brown bears in northern continental Eurasia belonged to two very closely related star-like mtDNA haplogroups based on cytochrome *b* (1140bp) sequences **(II)**: Eurasian (E) and Kamchatkan (K). These two haplogroups coexist only in Kamchatka Peninsula, whereas the majority of the area is covered by a single haplogroup (E). This is somewhat surprising as the area has a maximum east-west extent of 12 000 km. Results based on longer sequences (**II**, 1942bp) revealed four haplogroups: EA and EB were widely distributed, whereas EC and KA were specific to Estonia and Kamchatka, respectively (**II**).

Using terminology established by Leonard *et al.* (2000) all bears in northern continental Eurasia fall into subclade 3a, which is also present in Eastern Europe, Japan and Alaska. As all haplogroups (E, A, JA and K; **II**) in subclade 3a are very closely related, they are probably descended from a recent common ancestral population. Bears belonging to subclade 3a probably represent the most recent migration-wave that moved from Eurasia to Japan and Alaska. These bears therefore probably colonized Alaska after the LGM but before the appearance of the Bering Strait (approximately 11 000 BP; Elias *et al.* 1996). Bears may also have moved from mainland Eurasia into Hokkaido prior to approximately 12 000 BP ago, when a land bridge via Sakhalin Island existed (Bezverkhniy *et al.* 2002). The time of the MRCA of clade 3a (26 000 BP; brown bear sequences only, **II**) suggests that the expansion was recent and most likely occurred after the LGM. Considering the similar genetic distance between haplogroup E and the other haplogroups in subclade 3a, Kamchatka was probably occupied during the same migration-wave that reached Alaska and Japan, as Kamchatka was largely covered with ice during the LGM (Bigg *et al.* 2008), making the area unsuitable for brown bears at that time.

The current brown bear maternal lineage in Kamchatka is largely characterized by its own haplogroup. After the LGM a subset of the Eurasian brown bear population colonized Kamchatka. Individuals carrying the founder haplotypes (in haplogroup K) probably occupied most of the habitable areas rapidly, and doing so, they largely restricted further maternal gene flow between the mainland of Eurasia and Kamchatka Peninsula, as only single haplotypes of widespread haplogroup E (based on cytochrome b sequences) occur in Kamchatka. However, biparental markers group bears in Kamchatka and the Asian part of Russia into a single genetic group (Tammeleht *et al.* unpublished paper), highlighting the probable role of male-mediated gene flow in this area. Nonetheless, as sampling has been relatively scarce in the Asian part of Russia, additional sampling is required to better understand population processes in this area.

Moreover, there is some ambiguity concerning the distribution of subclade 3b: while this study recorded no 3b haplotypes in continental Eurasia, a single

bear carrying a 3b haplotype was recorded in the Russian Far East during an earlier study (Miller *et al.* 2006). Hence, more intensive sampling is required to clarify also the distribution of this subclade.

The results in paper **II** suggest that bear populations in northern continental Eurasia radiated out from a glacial refuge area (in Asia) after the LGM – as supported by the close relationship between all extant haplotypes – and that there was no significant geographic barrier that restricted the migration in the east-west axis of northern continental Eurasia. Moreover, similar phylogeographic patterns, with no substantial phylogeographic subdivision associated with reciprocal monophyly and signs of demographic expansion have been observed in a number of other mammals, but also in bird, reptile and plant species in northern Eurasia (e. g. the field vole (*Microtus agrestis*, Jaarola & Searle 2002), flying squirrel (*Pteromys volans*, Oshida *et al.* 2005), both pygmy and common shrews (*Sorex minutus* and *S. areneus*, Bilton *et al.* 1998), great spotted woodpecker (*Dendrocopus major*, Zink *et al.* 2002), adder (*Vipera berus*, Ursenbacher *et al.* 2006), mountain avens (*Dryas octopetala*, Skrede *et al.* 2006). Similarities found between these species suggest the presence of a general model for phylogeography in northern continental Eurasia: sudden expansion of a maternal lineage over the vast territory of northern Eurasia with no important barriers to dispersal. In the case of the field vole, fossil data indicate that the Ural Mountains may have represented the location of a single glacial refuge area in central Eurasia. Still, our data do not allow us to identify the precise location of this putative refuge area, similarly with many other studies based on contemporary mtDNA samples (e.g Ursenbacher *et al.* 2006). However, there are some species (namely the badger (*Meles meles*, Marmi *et al.* 2006), root vole (*Microtus oeconomus*, Brunhoff *et al.* 2003), collared lemming (*Dicrostonyx spp*., Fedorov *et al.* 1999) and common vole (*Microtus arvalis,* Haynes *et al.* 2003)) that show strong phylogeopgraphic structure across northern Eurasia and so do not fit into this general model of postglacial migration. Among these species, suture zones between generally distinct lineages are apparent near the Ural Mountains, which may represent a barrier for dispersal.

4.3. Divergence times of bear mtDNA lineages

It is widely accepted that brown bears and cave bears are sister-species, having evolved from *Ursus etruscus* (Bon *et al.* 2008; Kurtén 1976; Loreille *et al.* 2001). Based on paleontological evidence, the divergence of brown and cave bears has been estimated at 1.2–1.7 million years ago, whereas the oldest brown bear fossils are found in the territory of present-day China and dated about half a million years old (Kurtén 1968, 1976). Using multiple calibration our estimates of TMRCA of brown and cave bears (1.4 million years ago, **II**) was in good agreement with fossil data (it has to be noted, however, that this estimate

is heavily dependent on the single deep calibration point used). However, we also estimated the age of divergence without the fossil data, and this placed the divergence at only 266 000 years before present (BP; **II**). This is clearly an underestimate considering the results of other similar studies (Loreille *et al.* 2001; Hofreiter *et al.* 2002; Krause *et al.* 2008) and especially the fossil evidence (Kurten 1968). Such a result can be explained by the combination of time dependency of molecular rates and the selection of inappropriate calibration for this dataset (Ho *et al.* 2005; Ho & Larson 2006). If a split between species is studied but an estimate of the evolutionary rate is calibrated using internal calibration, then the extraspecific rate will normally be underestimated, as the short-term mutation rate is an order of magnitude greater than the long-term substitution rate (Ho & Larsson 2006; Ho *et al.* 2008; Henn *et al.* 2009). This is why we developed and applied a multiple calibration approach (**II**). This approach has recently been recommended for more general use, since increasing the number of calibrations will lead to more reliable results, as more accurate rate variation among lineages is obtained (Ho & Phillips 2009).

In article **II** we used a combination of radiocarbon dates at the tips and a fossil-based calibration at the root to account for the mixed intra- and interspecific nature of the dataset. Additionally, we used age priors, instead of point calibrations, yielding estimates with wider error intervals but probably with a more realistic reflection of the uncertainty related with the calibration (Ho & Phillips 2009). Using the multiple calibration approach we estimated that the MRCA of all sampled brown bears existed 193 000 BP (**II**), which is very close to the time to the estimate for European brown bears (~175 000 BP) using the internal calibration (**I**). It is likely that these estimates represent the same event, i.e. the MRCA of the sampled brown bear lineage as a whole is equivalent to the MRCA of the European brown bear lineage. However, earlier studies have estimated the time of MRCA of all brown bears to be three to five-fold older (see Davison *et al.* 2011 for details) (Taberlet & Bouvet 1994; Hofreiter *et al.* 2002; Talbot & Shields 1996). The large disparity between these estimates is mainly a result of differences in calibration (Ho *et al.* 2008b), as in earlier studies either external calibration or an imported substitution rate was used. It has been found that in analyses based on population-level sequences from multiple species or highly divergent conspecific populations, applying either external or internal calibration alone tends to yield biased date estimates (Gilbert *et al.* 2008). However, such calculations are entirely dependent upon the set of samples included for analysis. For example, by adding sequences and age data from two ancient polar bear samples to the dataset, Davison *et al.* (2011) obtained on average 40% older ages for some nodes compared to the respective estimations in **II**. The absence of extinct or otherwise unsampled taxa may result in date estimates that do not necessarily correspond to the origination times of the clades of interest. Hence, more representative sampling is needed to improve our understanding of the timing of evolutionary events.

The time of MRCA of clade 3a (26 000 BP; brown bear sequences only; **II**) coincides with the LGM and supports the suggestion that the expansion was recent and most likely occurred after the LGM. Again, as the formation time of European eastern lineage (24 420 BP, **I**) is very similar, these two estimates most probably represent the same evolutionary event.

When 95 complete mitochondrial genomes were sequenced (**III**) in north-western Eurasia to obtain highest matrilineal phylogeographic resolution possible, we identified five well supported phylogeographically distinct haplogroups and it was of great interest to know when these five haplogroups diverged. Divergence time estimation were performed based on fossil calibration (considering the divergence of cave and brown bears), as at that time no complete mitogenome sequences were available for ancient brown bears and for that reason internal calibration was not possible. Two separate analyses were performed to minimize the impact of purifying selection, which can influence age estimates of recent evolutionary events (Ho *et al.* 2005) and hence in first part of the analysis only third codon sites of protein-coding genes were used. The resulting estimate for the age of the MRCA of the 95 sampled brown bears, with a mean of 26 580 yr, was further used to estimate the MRCAs of the identified five haplogroups. These five major haplogroups were identified both with the network analysis and the Bayesian phylogenetic analysis, but as with BEAST these haplogroups obtained relatively low posterior probabilities, monophyly was enforced in further analysis with BEAST, and in this second part of divergence time estimation all three codon positions along protein-coding genes were used. In this second analysis the MRCA of analysed 95 brown bears was estimated as 20.4 ka. Still, as our analysis was actually reliant on a single fossil calibration (external calibration), the timings should therefore be treated with a degree of caution. The ages of the haplogroups varied more than twofold: the youngest being haplogroup D (specific to Finland), which formed about 7.7 ka ago; and the oldest being the primarily Russian based haplogroup A, which formed about 15.2 ka ago (**III**). The timing of these coalescence estimates imply that some genetic diversity within the population may reflect postglacial migration patterns. The large-scale westward migration from eastern European Russia towards Finland, apparent in the Bayesian phylogeographic analysis, is consistent with a pattern of recolonisation from a glacial refuge area located somewhere in Asia (**II**). However, based on our sample set it is not possible to identify the exact location and size of this refugium. As the haplogroups specific to Estonia and Finland (respectively, haplogroups B and D) are dated as the youngest they also correspond well with the time and direction of retreat of the Scandinavian Ice Sheet (SIS). During the LGM the SIS covered nearly all of Fennoscandia, northern Germany and Poland and large areas of the Baltic countries and after the LGM the ice sheet retreated in a south-east to north-west direction (Lambeck e*t al.* 2010). Hence, Finland was the last part of our study area to be free from ice. However, the retreating time of SIS exceeds the formation of bear lineages in Estonia and

Finland by thousands of years, and brown bear bone remains have been found in Estonia from the early Pre-Boreal (~9500 BP), indicating that brown bears recolonized the glacial landscape shortly after the retreat of the ice (Lõugas & Maldre 2000). It is interesting to note that the youngest and northernmost haplogroup (D; specific to Finland) formed about 7.7 thousand years ago, close to the beginning of the Atlantic, which was the warmest and moistest postglacial period, whereas the largest haplogroup (C) formed at approximately the time of the Younger Dryas, the coldest postglacial period. It is possible that during the cold postglacial periods part of the European brown bear population was again restricted to a refuge area in the east, while during the relatively warm periods some lineages were able to colonise the northern territories. Recent studies on field voles (Herman & Searle 2011) and least weasels (McDevitt *et al*. 2012) also discuss the importance of the Younger Dryas stadial in the divergence of European mammal lineages.

4.4. Complete mitogenomes – nucleotide diversity and selection

Complete mitogenome sequences provide the deepest possible insight into matrilineal phylogeography. Although short yet rapidly evolving (e.g. control region) segments of mtDNA can provide a coarse overview of the phylogeography of lineages over a wide area (e.g. Taberlet *et al.* 1998; Shapiro *et al*. 2004; Larson *et al.* 2005), such sequences harbor little or no phylogeographic signal in recently diverged lineages with low levels of mtDNA variation (as for example brown bears in northern continental Eurasia). In these cases, analysis of complete mtDNA can offer a considerable improvement in phylogeographic resolution.

Different regions of mtDNA evolve at different rates, leading to considerable variation in nucleotide diversity along the genome (**III**). Among the 95 mitogenomes sequenced in our study, most mutation hotspots were located in the control region, which is non-coding and thus able to accumulate frequent mutations without a significant cost to fitness. However, after removing the homoplasious regions, the nucleotide diversity of the control region dropped considerably, and the majority of other genes then exhibited higher diversity. However, the highest contributors to the NCmtDNA network were *ND5*, *ND4*, *CYTB* and the control region. On the other hand, when contribution was normalized in relation to gene length, the highest contributors were different (tRNAs, *ND6* and *ND4L*). It should be noted that when we constructed a network based on complete mitogenomes (*i.e.* including the full control region), the network structure was messy (containing many reticulations and median vectors), presumably due to the homoplasious characters. However, when homoplasious regions were removed, the network was well-resolved. Nevertheless, the contribution of sequence characters derived from the control region

was low in this network, as the other network based solely on protein coding genes was highly similar to the NCmtDNA network.

Despite the traditional view that mtDNA is selectively neutral, there is empirical evidence to challenge this assumption (e.g. Elson *et al.* 2004; Meiklejohn *et al.* 2007; Stewart *et al.* 2008). Nonetheless, positive selection is difficult to detect, as conservative genes often have more synonymous than non-synonymous substitutions, and even single amino acid changes can be adaptive if they are biochemically advantageous compared with the alternatives (Woolley *et al.* 2003; McClellan *et al.* 2005). For this reason, the widely used Ka/Ks ratio normally underestimates the role of positive selection (McClellan *et al.* 2005). Additionally, calculations of Ka/Ks ratio can be problematic when using closely related sequences, as a complete lack of synonymous sequences entails division with zero (Ingman & Gyllensten 2007); and this occurred with five genes in our dataset. To overcome these problems, we additionally used the program TreeSAAP, which has been successfully applied to detect positive selection in killer whale mitogenome data (Foote *et al.* 2010). TreeSAAP is not only able to accurately predict the presence of positive selection in given coding sequences, but it also identifies the specific properties of these regions, thus accurately defining a single gene's evolutionary descent. Both methods identified signs of positive selection in *ND6* gene, where one out of four non-synonymous substitutions were associated with changes in polarity, suggesting a possible functional change.

Among vertebrates *ND6* is the only gene that is encoded on the L-strand of the mtDNA. *ND6* is part of a large enzyme complex known as complex I (NADH dehydrogenase), which is necessary for oxidative phosphorylation. In humans, significant departures from neutrality in the *ND6* gene have been identified (Elson *et al.* 2004). Certain mutations in *ND6* gene are pathogenic and can cause diseases like Leber's hereditary optic neuropathy (e.g. Chinnery *et al.* 2001) or Leigh syndrome in humans (e.g. Ugalde *et al.* 2003). A study performed with domestic horses (*Equus caballus*) has revealed possible adaptive evolution in *ND6* among high-altitude animals, potentially influenced by oxygen deficits and cold temperatures (Ning *et al*. 2010). Positive selection of *ND6* in brown bears may therefore be associated with the ability to hibernate, though further analyses are needed to investigate this hypothesis.

SUMMARY

Phylogeography is a discipline that focuses on the principles and processes affecting the spatial distribution of genetic lineages, within and among closely related species. Phylogeographic data constitute important basic information underpinning the development of effective conservation and management strategies and for investigating historical processes acting on populations and species. Mitochondrial DNA (mtDNA), which is maternally inherited, has been the preferred molecular marker for phylogeographic analysis. Among wild mammals, the brown bear (*Ursus arctos*) has become a model species for phylogeographic analysis, due to wide distribution, the availability of numerous subfossils and its female philopatry. However, prior to the studies performed in the frame of this thesis, information on brown bear phylogeography was largely lacking for northern Eurasia, which nonetheless constitutes a major part of the species' current distribution and contains the majority of the world's brown bear population.

To fill this gap, the current thesis examined brown bear matrilineal phylogeography in northern Eurasia with particular focus on the population in northwest Eurasia. In brief, this thesis provides information about matrilineal phylogeographic patterns and demographic histories, postglacial recolonisation processes, divergence times of different brown bear lineages and haplogroups, and spatial processes acting on bear populations in northern Eurasia. It also provides an overview of brown bear mitogenomes: the genetic variation of different genes and non-coding regions, and genes under selection.

Several novel methods and approaches were developed. In papers **I** and **II**, divergence time estimates for different brown bear clades were calculated using novel molecular-clock calibration approaches, and in paper **III** a methodology was developed for sequencing the entire mitochondrial genomes of brown bears. Paper **III** additionally presented a novel spatially explicit individual-based method for identifying migration corridors and barriers. These new analytical methods are applicable for use in other studies with different species and populations.

The results suggest that all contemporary brown bears in northern Eurasia are relatively closely related and that during the Last Glacial Maximum (LGM) brown bears were most likely present in two refuge areas, one located possibly in central Europe – in the Carpathian Mountain range – and another in Asia, though the precise location is unknown. However, establishing the number and more exact locations for refuge areas requires further analysis. Our results also demonstrated that after the LGM, brown bears underwent a sudden demographic expansion and no significant geographic barrier was found to restrict migration across northern continental Eurasia. As this pattern has been previously described for other mammal species, it may represent a general model for the phylogeography of North-Eurasian mammals, as proposed in paper **II**. A second post-glacial expansion model for mammals was proposed in

the same paper: one where suture zones between genetically distinct lineages are apparent near the Ural Mountains, which may represent a barrier for dispersal.

Detailed analysis of the north-west Eurasian brown bear population using complete mitochondrial sequences (**III**) revealed five divergent, geographically confined and only partially overlapping haplogroups. Our analysis demonstrated that these five haplogroups formed during the post-glacial period and thus reflect a combination of postglacial migration patterns and more recent demographic history. A large-scale westward migration from eastern European Russia towards Finland was revealed, which is consistent with the results in paper **II** and suggested that a recolonisation radiated out from a glacial refuge area located somewhere in Asia. Potential migration barriers were revealed in Pskov oblast (Russia) and south-eastern Finland, while a migration corridor was found in western Russia. Ninety-five complete mitogenomes, sequenced during this study, allowed us to make the first analysis of intra-specific variation among brown bear mitogenomes. Positive selection was found to act on the gene *ND6*.

The results of this study provide scientific data that is potentially very useful for the development of effective management and conservation strategies for Eurasian brown bears in the near future. However, for sound conservation and management decisions, additional data from autosomal and Y-chromosome genetic loci would be of great benefit as these can provide further insight into the population processes of brown bears in northern Eurasia.

SUMMARY IN ESTONIAN

Pruunkaru (*Ursus arctos*) fülogeograafia Põhja-Euraasias

Fülogeograafia on valdkond, mis on keskendunud liigisiseste ja lähiliikidevaheliste geneetiliste liinide ruumilist paiknemist mõjutavate seaduspärasuste ja protsesside uurimisele. Fülogeograafilised andmed on oluliseks lähteinformatsiooniks efektiivsete kaitse- ja majandamisstrateegiate väljatöötamiseks, aga ka fundamentaalteaduslikust aspektist, uurimaks populatsioone ja liike mõjutanud ajaloolisi protsesse. Fülogeograafilistes analüüsides on seni geneetilise markerina eelistatud emaliini pidi edasikanduvat mitokondriaalset DNA-d (mtDNA). Looduslike imetajaliikide hulgas on olnud üheks mudelliigiks pruunkaru, seda eelkõige tänu laiale levikule, arvukatele luuleidudele ja emakarude filopatrilisusele. Siiski oli Põhja-Euraasia kohta käiv fülogeograafiline informatsioon seni puudulik, hoolimata asjaolust, et see piirkond moodustab enamiku antud liigi tänapäevasest levikualast ning hõlmab suure osa maailma karupopulatsioonist.

Mainitud tühimiku täitmiseks vaatleb käesolev doktoritöö pruunkaru emaliini fülogeograafiat Euraasias, pöörates erilist tähelepanu Loode-Euraasia populatsioonile. Lühidalt kokkuvõetuna: antud doktoritöös käsitletakse pruunkaru emaliinide fülogeograafilisi mustreid ja demograafilist ajalugu, jääajajärgseid rekolonisatsioonisuundi, erinevate pruunkaruliinide ja haplogruppide lahknemisaegu ning ruumilisi protsesse Põhja-Euraasia pruunkaru populatsioonis. Samuti antakse ülevaade pruunkaru mitokondri genoomist: geneetilisest variatsioonist erinevates geenides ja mittekodeerivates regioonides ning selektsiooni all olevatest geenidest.

Välja töötati mitu uut meetodit ja rakendust. **I** ja **II** artiklis rakendati uudset molekulaarse kella kalibreerimist erinevate pruunkaruklaadide lahknemisaegade arvutamiseks ning **III** artikli raames töötati välja metoodika pruunkaru mitokondri täisgenoomide sekveneerimiseks, ning uudne indiviidipõhine ruumilisgeneetiline meetod migratsioonikoridoride ja -takistuste väljaselgitamiseks Loode-Euraasia karupopulatsioonis. Need uued meetodid on edaspidi laialdasemalt rakendatavad teistegi liikide ja populatsioonide puhul.

Antud töö tulemused viitavad, et tänapäeval on kõik pruunkaruliinid Põhja-Euraasias suhteliselt lähedalt seotud ja et tõenäoliselt olid pruunkarud viimase jääaja maksimumi ajal esindatud vähemalt kahes refuugiumis: üks asus arvatavasti Kesk-Euroopas Karpaatia mäestikualal ja teine Aasias, kuid selle täpsem asukoht on seni teadmata. Refuugiumite täpsema arvu ja asukoha väljaselgitamine nõuab kindlasti edasist analüüsi. Töö tulemused näitavad muuhulgas, et peale viimase jääaja maksimumi toimus tugev demograafiline ekspansioon ning ükski geograafiline tõke ei olnud piisavaks takistuseks pruunkarude migratsioonile üle Mandri-Euraasia põhjaosa. Et antud mustrit on eelnevalt kirjeldatud ka teiste imetajaliikide puhul, siis pakuti **II** artiklis, et see võiks esindada Euraasia imetajate fülogeograafias üldlevinud mudelit. Lisaks

esitati samas töös ka teine imetajate jääajajärgse ekspansiooni mudel, kus geneetiliselt eristunud liinide vaheline kontakttsoon asub Uurali mäestiku lähedal, mis kätkeb endas potentsiaalset levikubarjääri.

Loode-Euraasia pruunkaru populatsiooni detailne analüüs mitokondri genoomi täisjärjestuste alusel (**III**) selgitas välja viis selgesti eristatavat, geograafiliselt piiritletud ja ainult osaliselt kattuvat haplogruppi. Analüüsid näitasid, et need viis haplogruppi moodustusid jääajajärgsel perioodil ning peegeldavad seega kombinatsiooni jääajajärgsest migratsioonimustrist ja hilisemast demograafilisest ajaloost. Ilmsiks tuli suur läänesuunaline migratsioon Euroopa-Venemaa idaosast Soome suunas, mis on kooskõlas **II** artikli tulemustega, et jääajajärgne kolonisatsioon lähtus arvatavasti kusagil Aasias paiknenud refuugiumist. Potentsiaalne migratsioonitõke tuvastati Pihkva oblastis (Venemaal) ja Kagu-Soomes, samas kui Lääne-Venemaal tuvastati migratsioonikoridor. Et selles töös sekveneeriti 95 mitogenoomi, siis võimaldas see esimest korda analüüsida ka pruunkaru mitokondri genoomide liigisisest variatsiooni. Positiivne evolutsiooniline valik tuvastati valgugeenile ND6.

Antud töö tulemused pakuvad olulist baasinformatsiooni pruunkaru efektiivsete majandamis- ja kaitsestrateegiate väljatöötamiseks Euraasias. Samas on lõplike otsuste langetamiseks lisaks mtDNA-andmetele kindlasti vaja kaasata ka autosomaalsete kromosoomide ja Y-kromosoomi andmed – need pakuks lisaks emaliiniga seotud protsessidele olulist täiendavat teavet.

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PUBLICATIONS

CURRICULUM VITAE

Name: Marju Keis
Date of birth: August 11, 1981
Citizenship: Estonian
Contact: Department of Zoology, Institute of Ecology and Earth Sciences, Vanemuise 46, 51014, Tartu, Estonia
E-mail: marju.keis@ut.ee

Educational history:

1996–1999	Tartu Mart Reiniku High School
1999–2003	University of Tartu, *baccalaureus scientarum* in zoology
2003–2006	University of Tartu, *magister scientarum* in zoology
2004–2005	University of Tartu, profession of biology teacher
2006–2013	University of Tartu, doctoral studies in zoology

Professional employment:

2005	Rakvere Private High School, biology and geography teacher
2008	University of Tartu, Institute of Ecology and Earth Science, specialist
2012	University of Tartu, Institute of Ecology and Earth Science, researcher

Publications:

Keis, M., Remm, J., Ho, S.Y.W., Davison, J., Tammeleht, E., Tumanov, I.L., Saveljev, A.P., Männil, P., Kojola, I., Abramov, A.V., Margus, T., Saarma, U. 2012. Complete mitochondrial genomes and a novel spatial genetic method reveal cryptic phylogeographic structure and migration patterns among brown bears in north-western Eurasia. *Journal of Biogeography*, doi:10.1111/jbi.12043.

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Nimi: Marju Keis
Sünniaeg: 11.08.1981
Kodakondsus: Eesti
Aadress: Ökoloogia ja Maateaduste Instituut, Zooloogia osakond, Vanemuise 46, 51014, Tartu, Eesti
E-post: marju.keis@ut.ee

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1999–2003	Tartu Ülikool, *baccalaureus scientarum* zooloogia erialal
2003–2006	Tartu Ülikool, *magister scientarum* zooloogia erialal
2004–2005	Tartu Ülikool, bioloogiaõpetaja kutse
2006–2013	Tartu Ülikool, zooloogia doktorantuur

Teenistuskäik:

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2008	Tartu Ülikool, Ökoloogia- ja Maateaduste Instituut, zooloogia õppetooli spetsialist
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