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Authors: Iso-Touru, Terhi, Tabell, Jonna, Virta, Anneli, and Kauhala, Kaarina

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Terhi Iso-Touru, Jonna Tabell, Anneli Virta and Kaarina Kauhala

T. Iso-Touru (https://orcid.org/0000-0001-8258-9047) ✉ (terhi.iso-touru@luke.fi), J. Tabell, A. Virta and K. Kauhala, Natural Resources Inst. Finland (Luke), Finland.

For the ability to control an invasive species and to protect an ecologically similar native species it is essential to map the exact distributions of both species. This is difficult if the species are so morphologically similar that their identification in the field is almost impossible. In Finland, the invasive North American beaver *Castor canadensis* is spreading towards the range of the native Eurasian beaver *Castor fiber* and at present, these species are partly sympatric. Effective management of these morphologically similar species requires an efficient method for species identification, ideally one that is non-invasive. Non-invasive genetic methods are used in a wide variety of wildlife species, for example in the research of large carnivores. Feces are a good source of DNA for terrestrial animals, but for the semi-aquatic animals like beavers, feces are not the best option. However, environmental DNA (eDNA) has been successfully used to detect species non-invasively in aquatic and terrestrial environments.

We developed a non-invasive, eDNA-based method to map the distribution of the beaver species in Finland and to investigate within-species genetic diversity. The eDNA was obtained from the feed remains (wood chips) from beaver forage sites. With the help of Citizen science, wood chip samples were collected from different parts of Finland. We used our eDNA method to identify the ranges of both beaver species. Additionally, the presence of Eurasian beavers in south-east Finland was proven for the first time. Our non-invasive eDNA method is an effective way to accurately identify the ranges of both beaver species and will allow for the control of the invasive North American beaver and conservation of the native Eurasian beaver in Finland.

Keywords: eDNA, Eurasian beaver, mtDNA, non-invasive, North American beaver, species identification

Invasive species are recognized as one of the worst threats to biodiversity (Ebenhard 1988). When the ranges of ecologically similar invasive and native species approach each other, it is important to control the spread of the invasive species. Therefore, it is essential to map the exact distributions of both species. The method should preferably be non-invasive so that no individuals of the species are disturbed by the procedure. Traditional methods to collect samples for DNA extraction (blood, tissue or hair sample) typically require the capture of the individual. This is not only stressful for the animal but also demanding to implement in field. The use of a non-invasive method is particularly important in the case of an endangered native species.

The Eurasian beaver *Castor fiber* is classified as a near threatened species in Finland (Hyvärinen et al. 2019). The original Eurasian beaver was extinct in Finland in late 19th century but has recovered due to releases of 19 individuals from Norway in the 1930s (Lahti and Helminen 1969, 1974, Lahti 1972). Unfortunately, at least seven individuals of North American beaver *C. canadensis* from the USA (Parker et al. 2012) were also released into several places in southern and central Finland, because at that time its status as a distinct species was not recognized. The Eurasian beaver disappeared from places where both species were introduced, and its population grew slowly only in the region of Satakunta, south-west Finland (Lahti and Helminen 1980). Its present distribution covers areas in south-west Finland (mainly Satakunta) and western Lapland where beavers probably wandered from Sweden (Fig. 1, Iso-Touru et al. 2020). In addition, some Eurasian beavers likely migrated to south-eastern Finland from Russian Karelia where a Eurasian beaver population exists (Danilov et al. 2011a). The introduced, invasive North American beaver population increased much faster and soon occupied most of eastern and central Finland (Lahti and Helminen 1969). It also occurs sporadically in Lapland, because some were relocated from southern Finland to Lapland (Lahti 1972).
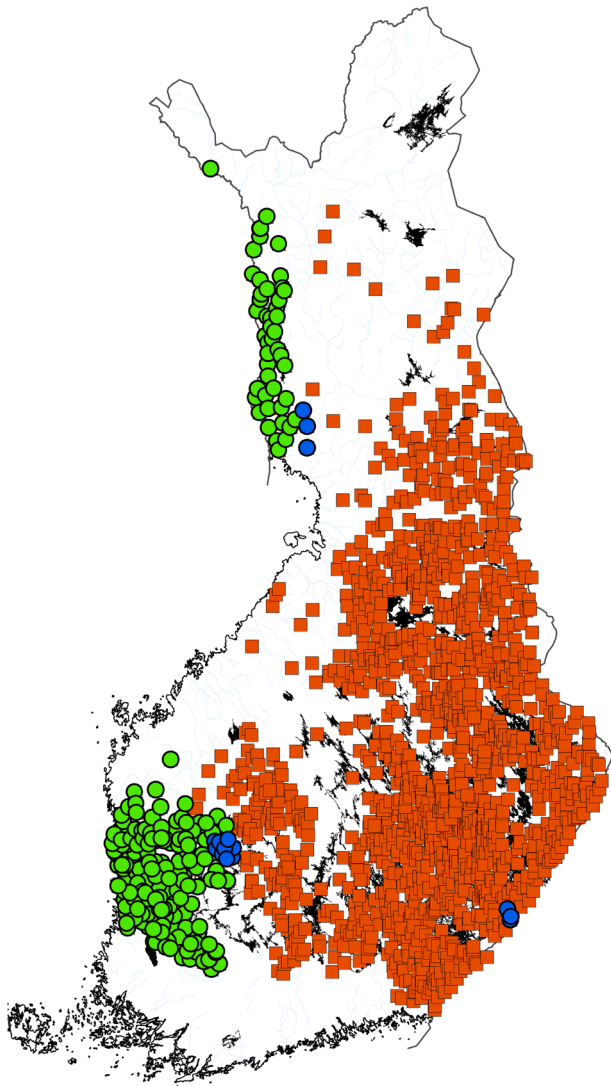
Figure 1. The distribution of the native Eurasian beaver and the invasive North American beaver in Finland based on the monitoring count in 2020. Identification of species was mainly estimated from lodge location but information from DNA analyses of wood chips was also used. For monitoring counts, see Brommer et al. (2017). Green bullets = Eurasian beaver, brown squares = North American beaver, blue bullets = areas where both species occur. Original figure: Natural Resources Institute Finland, Luke.

In recent years, North American beavers have spread towards the range of the native species in Finland and at present the two species are sympatric in at least three river systems (Kauhala and Karvinen 2018). Because the two beaver species are fairly similar in their habitat use, it is possible that they compete for the best habitat patches with deciduous trees (Kauhala and Turkia 2013, Alakoski et al. 2019). The invasive species has larger litters (Parker et al. 2012), which may be responsible for the faster growth rate of the population after introductions and thus probably also in areas where the species is spreading at present. In the worst case, it may threaten the future of the native beaver in Finland.

Management for beavers in Finland should include accurate mapping of distributions of both species. Reliable identification of beaver species in the field is difficult

or impossible because the two species are morphologically very similar. Species can be identified on the basis of skull morphology from dead individuals (Danilov et al. 2011b, Kauhala and Timonen 2016) but the need for the non-invasive (genetic) identification method is obvious. Non-invasive genetics has become an important monitoring tool for example in the research of large carnivores (Kojola et al. 2018). For terrestrial animals, feces are a good source of DNA, but for the semi-aquatic animals like beavers, feces are not the best option.

Environmental DNA (eDNA) has been successfully used to detect species non-invasively in aquatic and terrestrial environments (Deiner et al. 2017). Typically, the environmental origin of the eDNA sample is water, sediment or soil (Pawlowski et al. 2020a). However, other eDNA sources, such as feed remains, may offer equally good source of eDNA. Maternally inherited mtDNA is often used in eDNA studies because it has greater number of copies per cell than nuclear DNA and the detection rate is assumed to be higher even where DNA is present at a low concentration and/or is degraded (Rees et al. 2014, Miya et al. 2015). It is used to study both intraspecific (Tapio et al. 2006, Frosch et al. 2014, Kvist et al. 2019) and interspecific relationships (Kumar et al. 2016). In single species monitoring studies, the eDNA is normally amplified with species-specific primers (Strand et al. 2019) using either quantitative PCR or digital PCR (Pawlowski et al. 2020b) and the length of the DNA amplicon is typically less than 300 bp to overcome the problem of DNA degradation.

For beavers, wood chips left at bases of trees around the lodge sites offer a non-invasive source of DNA that can be used to reveal the beaver species living in the area. Thus, the aim of this study was to develop a non-invasive, DNA-based method for beaver species identification and to map the detailed distribution of the two beaver species in Finland, especially in areas where the species are sympatric, live close to each other, or where the distribution is poorly known (Lapland). We developed a method to extract DNA from wood chips and used a part of the hypervariable domain of the control region (D-loop) of mitochondrial DNA (mtDNA) to distinguish beaver species. We chose to amplify a longer DNA fragment compared to typical eDNA studies. Also, unlike typical eDNA studies, we amplified the DNA by standard PCR and identified the species by sequencing the obtained DNA fragment by Sanger sequencing. Jo et al. (2017) showed that in aquatic environment, using longer DNA fragments may reveal more contemporary biological information. In addition, we were able to study interspecific genetic diversity with our approach.

Material and methods

Wood chip samples from the foraging sites of the beavers were collected by volunteers. When beavers gnaw and fell trees, cells from the oral mucosa stick to the wood chips enabling extraction of the DNA.

Instructions on how to collect the samples were given on the web page of Natural Resources Institute Finland (Luke) (<www.luke.fi/tietoa-luonnonvaroista/riista/majavat/maja-vanaytteiden-kerays-2/>) and the information was spread

also by the Finnish Wildlife Agency and hunting organizations. Briefly, volunteers were asked to collect samples (incl. at least 10 wood chips/sampling site) from recently used beaver foraging sites and to wear disposable gloves while collecting the samples. They were asked to give the exact location of the samples (ETRS-TM35FIN coordinates) and keep samples at +8°C prior to sending them to Luke. In the laboratory, samples were kept at +8°C, and for long-term storage, stored at −20°C. A reference wood chip sample of North American beaver was obtained from Ähtäri Zoo, Finland.

DNA extraction

A total of 258 wood chip samples from different locations were processed (Supporting information). The wood chips were handled in a laminar flow cabinet (Kojair Tech Oy) placed in a separate laboratory. On average, 5 g of wood chips were used per sample. The DNA was collected using the 4N6FLOQSwabs for Crime Scene (Copan Diagnostics). First, a drop of sterile water was placed on the side of the swab tip. The moistened swab tip was rotated on the surface of woodchips, inserted in a 2 ml Eppendorf tube and the shaft was broken from the breaking point. To precipitate the DNA, 75 µl of 3 M NaOac (pH 5.2) and 1.65 ml ethanol were added to the tube. The tube was mixed 20 times by turning it up and down and vortexed briefly. DNA precipitation was allowed to occur overnight at −20°C. Next day, tube with the swab was vortexed for 5 sec and centrifuged (18 000 g, 1min at 5°C). After the first centrifugation step, the swab was removed with sterilized forceps. The remaining fluid was centrifuged (18 000 g, 30 min at 5°C). After centrifugation, the supernatant was removed. The DNA was extracted from the remaining pellet using DNeasy blood and tissue kit (Qiagen), following the manufacturer's instructions with the following modification: the final elution was done to 40 µl of elution buffer.

PCR amplification and sequencing

The PCR was conducted with DreamTaq DNA Polymerase in a 15 µl volume of 1× PCR buffer, 0.2 mM dNTPs, 10 pmol primer mix (forward primer: 5′-TCCACACAT-CAAAACAACGAA-3′ and reverse primer: 5′-GGCCCT-GAAGTAAGAACCAG-3′) and 1–4 µl of DNA elution. The primers amplify approximately 840 bp region of the mtDNA D-loop from both the species. The PCR cycling conditions were following: 1) an initial denaturation at 95°C for 3 min, 2) 40 cycles of 30 s denaturation (94°C), 30 s hybridization (63°C), 30 s elongation (74°C) and a 3) final 3 min elongation (74°C). DNA samples extracted with the Qiagen blood and tissue kit from the Eurasian and North American beaver tissues were used as controls.

PCR products were separated on a 1% agarose gel. For samples that amplified poorly, the DNA elution was concentrated by evaporation and diluted again to 10 µl of sterile MQ water. One to four microliter of concentrated DNA elution was used to perform the new PCR amplification following the PCR conditions described above.

After successful amplification, samples were purified and directly sequenced by Sanger sequencing using the BigDye Terminator Cycle Sequencing Kit. Electrophoresis of sequencing reactions was performed on 3500xL Genetic Analyzer, and sequences were visually inspected and aligned with the reference sequences obtained from the GenBank from both Eurasian beaver and North American beaver with Sequencher 5.4.6. The species were determined based on the alignments and confirmed with the BLAST searches done with NCBI nucleotide BLAST (dbV4). Samples (n=99 for Eurasian beaver and n=69 for North American beaver) that yielded 500 or more good quality base pairs were used to construct mitochondrial haplotypes for both the species separately. DnaSP ver. 6.12.03 (Rozas et al. 2017) was used to construct haplotypes. Median joining network (Bandelt et al. 1999) was built with Network 10.1.0.0 to illustrate relationships and frequencies among the haplotypes for both the species.

Results and discussion

We were able to identify the species from 83% of samples (n=213, 143 Eurasian beaver samples and 70 North American beaver samples). As our primer pair was not species-specific but amplified both Eurasian and North American beaver mtDNA, we were able to identify species from one PCR/sequencing reaction. This reduces costs and ensures that the limited amount of DNA available is sufficient. However, approximately half of the successful samples were processed at least twice (starting from DNA extraction or PCR) before species was identified successfully. In general, DNA degradation due to high temperature, UV light and/or rain/snow together with microbial contamination poses challenges in eDNA studies. Also, good sampling and laboratory practices are necessary to ensure contamination-free end products (Pawlowski et al. 2020b). The sampling was done via citizen science. This can introduce uncertainty, but we tried to overcome the issue by providing good sampling protocol with the local language and spreading information through the Finnish Wildlife Agency. To avoid contaminations in the laboratory, we used separated laboratory and a laminar flow cabinet for sample handling. Also, samples were sequenced only if the visible band of correct length (~840 bp) was seen in an agarose gel (Supporting information).

Since the amplified DNA fragment was long, besides identifying species, we were also able to construct long mtDNA haplotypes and study interspecific genetic diversity of the two beaver species. The mtDNA haplotype sequences observed in this study from the *Castor fiber* and *C. canadensis* samples have been deposited to GenBank (accession numbers MZ262277–MZ262287). Figure 2 shows the geographic distribution of the *C. fiber* and *C. canadensis* haplotypes and the genetic relationship and frequencies of the detected haplotypes.

It was not known earlier that Eurasian beavers exist in south-east Finland (Fig. 2a, c, haplotypes Cfib5 and Cfib6). They most likely originate from Russian Karelia where Eurasian beavers are known to occur and are suspected to have migrated to south-eastern Finland (Danilov et al. 2011a). The migration route is supported by the observation that Eurasian beavers in south-east Finland belong to different maternal haplogroups than Eurasian beavers in other parts of Finland (Fig. 2a, c). Since there is little genetic variation in *C. fiber*
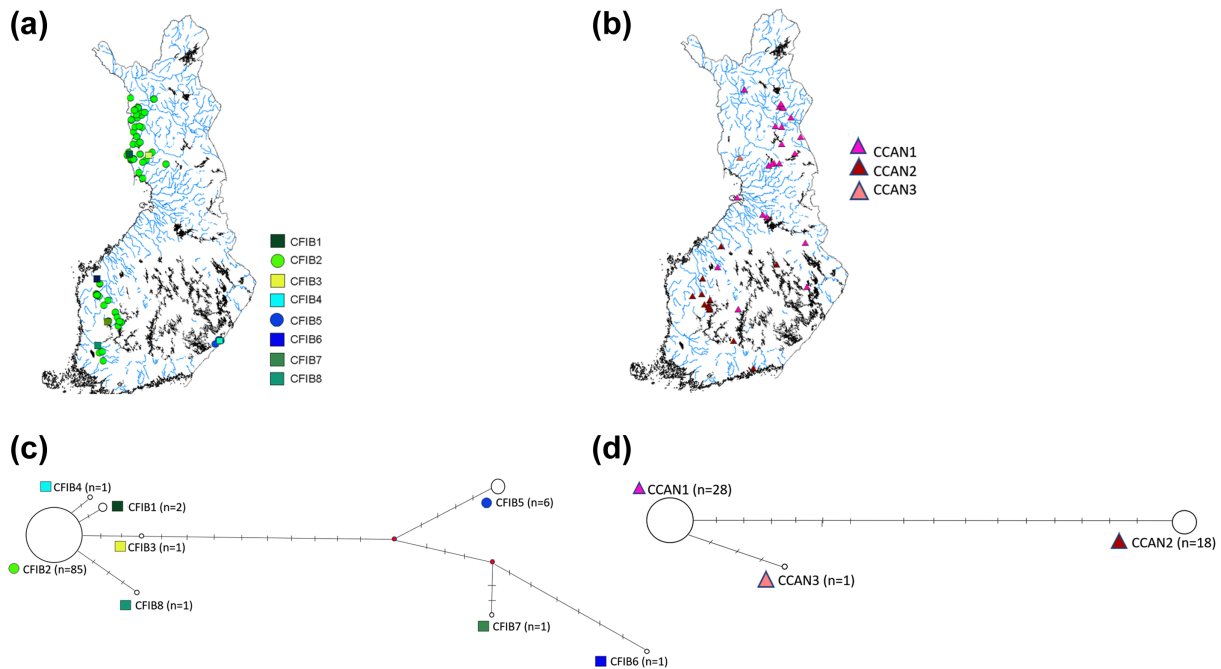
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Figure 2. Geographic distribution of the (a) *C. fiber* and (b) *C. canadensis* haplotypes. Different haplotypes are shown as differently colored squares, circles and triangles. Median joining haplotype network (created by Network 10.1.0.0) is given separately for *C. fiber* (c) and for *C. canadensis* (d). The number of haplotypes observed are given in (c) and (d) in parentheses after the haplotype name. Mutated positions are shown as vertical lines and node sizes are proportional to frequencies. Median vectors (mv1 and mv1 (haplotypes not present in the current data)) are shown in red.

population in Finland compared to the population in Russia (Iso-Touru et al. 2020), the immigrants from Russia could improve the situation. Translocation of some *C. fiber* individuals from south-east Finland to its main distribution area in south-west Finland to increase the genetic variation of the population should be considered. Unlike other haplotypes in south-west Finland, the haplotype Cfib7 (Fig. 2a) is genetically closer to the haplotypes found in south-east Finland than those found elsewhere in the country (Fig. 2c). In this study, the origin of the haplotype Cfib7 remains unclear. The low frequency *C. fiber* haplotypes Cfib1 and Cfib3 (Fig. 2c) in Lapland may have spread with beavers that migrated from Sweden.

Wood chips were mostly obtained from regions populated by Eurasian beavers. This might partly explain the low amount of *C. canadensis* haplotypes observed in this study. Furthermore, only about seven individuals of *C. canadensis* were released, (Lahti 1972) and may have resulted in low genetic diversity due to a founder effect. If more samples were obtained from the Eastern part of Finland (Fig. 1), it is possible that more mtDNA haplotypes for the North American beavers may have been found. Unlike in Finland, North American beavers were never relocated to other Scandinavian countries. Live-trapped North American beaver individuals in Finland were released in the 1940s near the Russian border (Lahti and Helminen 1974). Similar releases of North American beavers were conducted in Karelia. The North American beaver population in Karelia has grown and is assumed to have spread north along the Finland-Russian border (Parker et al. 2012).

This study confirmed earlier identifications of the beaver species from skull morphometry and some DNA

analyses from tissue samples of hunted beavers (Kauhala and Timonen 2016, Iso-Touru et al. 2020) but also provides new information of the distribution of beavers from a larger area. Furthermore, all recent species identifications from Lapland are based on this study, i.e. DNA analysis from wood chips. Beavers have not been hunted in Lapland (so no skulls or tissue samples were obtained from Lapland) in recent years because the distribution of Eurasian beaver there was not known, and hunters need a license to hunt Eurasian beavers in Finland. Therefore, hunters did not want to take the risk that they would illegally kill a Eurasian beaver in Lapland. Our new non-invasive DNA method now enables beaver species identification throughout Finland and thus promotes the control of the invasive beaver and conservation of the native beaver in Finland. It has been suggested that North American beavers should be removed from areas where the two species are sympatric or live close to each other (National Management Plan for Beavers, in prep.). Using this eDNA method the ranges of beaver species can be accurately identified in the critical areas where beaver species are sympatric or live close to each other. This helps selective hunting of the invasive species.

Conclusions

We present here a non-invasive DNA based method to identify beaver species from environmental samples. The method uses wood chips that beavers leave at the foraging site. The wood chip samples are easy to collect and simple to store before processing. The developed extraction method can be

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used to monitor the detailed distributions of the two beaver species in Finland. Precise monitoring will help to control the spread of the invasive beaver and to enable the spread of the native beaver in country. Our method is easily applied to monitor species that leave feed remnants that can be collected in the field. Feed remnants are an option to use eDNA and monitor (semi-aquatic) animals that do not leave feces which are easily found. The method can be used not only for species identification but potentially also to identify individuals by using multiple genetic markers.

Author contributions

KK and TIT designed the study, JT and AV developed the extraction method and genotyped the samples. TIT and KK analyzed the data. TIT and KK wrote the manuscript. All authors read and approved the final manuscript.

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