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Master Thesis

Development of microsatellite panels for determining genetic structure in populations of pine marten *(Martes martes)*

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Abstract

The population genetic structure of the Norwegian pine marten (*Martes martes*) is poorly understood. Genetic study of this species is challenging due to their elusive nature and limited available information. In the present study, samples were collected by using non-invasive genetic techniques and genotyping individual pine marten using microsatellite markers. The objectives of my study were primarily to develop microsatellite panel from the microsatellite markers previously used for other mustelid species and secondarily to evaluate DNA extraction methods from different biological samples. DNA was successfully extracted from fresh muscle tissue, hair and dry skin with fur (samples from museum specimen). However, the quality of DNA extracted from hair samples and museum specimen samples was considerably low. For the development of the microsatellite panels, 18 microsatellite markers were successfully grouped into 3 PCR multiplex panels including one sex identifying marker containing 4-8 microsatellite loci in each multiplex panel. One of the fresh muscle tissue sample successfully amplified 100 % microsatellite loci used in this study. Out of the other remaining 6 tissues including frozen tissue samples analyzed, 79 % of the microsatellite loci were amplified on genotyping while only 32 % of the microsatellite loci were amplified in 8 hair samples. On the other hand, museum specimen sample did not amplify microsatellite loci at all. The genotyping success rate of hair samples was found to be significantly lower compared to tissue samples. However, due to a smaller size of hair samples and degradation of DNA extracted from hair samples, I could not tests sufficient to draw a clear conclusion about genotyping success rate of hair samples. The genotyping result showed that all the microsatellite loci had variation in alleles ranging from 2 to 11 alleles per locus. The ISOLATE-II Genomic Kit and the Chelex 100 protocol yielded amplifiable DNA from tissues and hair samples respectively among the methods used for the DNA extraction. In conclusion, this developed microsatellite panel of microsatellite markers and PCR protocol can be used further for the population genetics study of pine marten in Norway.

Keywords: Microsatellite markers, PCR, multiplex panel, Genotype, Pine marten

Table of Contents

1. Introduction

In the past decades, non-invasive genetic sampling have proven to be a convenient method for studying elusive species that are difficult to sample using traditional methods such as live capture and direct sightings (Messenger & Birks 2000; Ruiz-González *et al.* 2008). Genetic sampling can be used to obtain data on space use and population density, which are particularly difficult to obtain for elusive carnivores like pine marten with little information on population structure (Manzo *et al.* 2012; O'Mahony 2014; Balestrieri *et al.* 2016a).

Hair and scats are the most commonly used non-invasive biological samples. DNA is isolated from these samples and tested using various genetic markers. Hairs with follicles typically yield a better quality of DNA than scats, however, a single hair usually yields much lower DNA than scats, but multiple hairs can increase DNA yield (Kelly *et al.* 2012). Genetic markers such as microsatellites, single nucleotide polymorphisms (SNP's), mitochondrial DNA (mtDNA), 16S rDNA, 18S rDNA and allozymes can be used to estimate various parameters of interests. The most common parameters are population genetic structure, demographic bottlenecks, kinship, relatedness, phylogenetic lineage and gene flow (Mowat & Paetkau 2002; Pearse & Crandall 2004; Manel, Gaggiotti & Waples 2005; Kelly *et al.* 2012; Morin, Kelly & Waits 2016). Thus, the application of genetic sampling is rapidly becoming a more widespread, efficient, valuable and powerful tool for establishing effective management plans in wildlife conservation (Selkoe & Toonen 2006; Kristensen *et al.* 2011; Rodgers & Janečka 2013). Selkoe and Toonen (2006) reviewed publications on microsatellite markers and proposed useful steps for a microsatellite screening protocol for quality testing of data sets. Further, the development of advanced technology and new software programs have facilitated powerful and efficient genetic analyses that could inspire ecologist to apply genetic tools (Pearse & Crandall 2004). Still, clear protocols on the development of microsatellite markers are lacking for many taxa, as well as bioinformatic tools for primer design and an allele calling (Guichoux *et al.* 2011).

1.1 Pine marten (*Martes martes*)

The European pine marten (*Martes martes*, L. 1758) is a medium sized woodland-dwelling mustelid (Proulx *et al.* 2005; Mullins *et al.* 2010). The pine marten has a wide distribution in the west and central Palaearctic across the European continent, with a range encompassing the

Mediterranean (Italy) to Fennoscandian taiga (Upper borderline) and the western Siberia (Asian Russia), Iran and Asia (Proulx *et al.* 2005). Pine martens inhabit a variety of habitat types including high alpine mixed shrublands with conifers to lowland deciduous forests (Fornasari *et al.* 2000). More rarely, they are also found in woodlands with incomplete canopy and dense understory vegetation (Proulx *et al.* 2005). The species is listed in the category "least concern" in the IUCN Red list of Threatened Species (Hoffmann, Duckworth & Conroy 2008). Habitat fragmentation and overharvesting has high influence on demography and population (Mullins *et al.* 2010; Mergey, Helder & Roeder 2011). Population distribution and temporal variation are also influenced by climate, interspecific interactions and food availability (Balestrieri *et al.* 2016a). Moreover, mountain ranges may acts barriers for gene flow limiting their dispersal, density, evolutionary potential and may lead to higher risk of local extinction (Caro & Laurenson 1994).

1.2 Phylogeographic studies of pine marten

The theme of phylogeography is concerned with the principles and processes governing geographic distributions of genealogical lineages over time and space particularly within closely related species and higher taxa (Yang, Dong & Lei 2009). Some studies have tried to get insight into the phylogenetic history and genetic variability of the pine marten based on DNA (Schwartz *et al.* 2012; Pertoldi *et al.* 2014). Two separate mitochondrial DNA fragments; the control region or displacement (D) loop and cytochrome *b* (cyt *b*) were used by Davison *et al.* (2001) for quantifying phylogeography of *M. martes in Europe.* Study suggests that all the populations in central and northern Europe may have been colonized from an Iberian and Balkans refugia (Sommer & Benecke 2004). A more recent study using the cytochrome *b* gene, tRNApro, tRNAThr, the control region (d-loop) and 12S ribosomal RNA (rRNA) indicates that the phylogeographic history of *M. martes in* northern Europe has a mixed pattern of recolonization both from a Mediterranean and a non- Mediterranean. This suggest that most of the European population of pine marten was likely colonized by the central-northern European phylogroup (Ruiz-González *et al.* 2013) with the ongoing expansion of Alpine and trans-Alpine pine marten population (Balestrieri *et al.* 2016a).

1.3 Populaton genetic structure

Population genetics is the theory describing the evolution of genetic makeup of a population of similar organisms as a result of selection, mutation, migration and genetic drift (Ewens 2012; Singh 2015).These study often uses highly variable DNA markers such as nuclear microsatellites for evaluating contemporary patterns of genetic variability and gene flow within a species (Schwartz *et al.* 2012). Such genetic data can be used for various purposes such as to define substructure, identify isolated populations and define units of conservation (Schwartz *et al.* 2012). A study on 270 European pine martens across the Netherland found no genetic diversity (de Groot *et al.* 2016). Similar studies on *M. martes* in the current distribution area in Europe (Kyle, Davison & Strobeck 2003) and other Martes species; *M. americana* in Canada (Kyle, Davis & Strobeck 2000) have observed a low level of genetic structure in Europe(Kyle, Davis & Strobeck 2000). In addition, the study using allozyme electrophoresis also indicated high variability in a population of *M. Americana* in the central Rocky Mountains forest of Wyoming, United States (Mitton & Raphael 1990).

On the other hand, a measure of the effective population size (N_e) is a common tool for monitoring populations, which is necessary for evaluating the mechanisms of genetic differentiation in natural populations (Husband & Barrett 1992; Schwartz *et al.* 2012). The effective population size gives an idea on how a real population should be affected by inbreeding and genetic drift relative to an ideal population (Crow & Kimura 1970; Buskirk & Ruggiero 1994). Therefore, knowledge about demography and life history traits, including sex ratio, population size over time, variation in reproductive output among individual in their lifetime are required to calculate inbreeding N_e (Crow & Kimura 1970). In a population, small N_e indicates more genetic drift; hence reduces its potential to adapt to environmental changes, while the populations with larger N_e indicate high genetic variability is useful for adaptation into the future. Thus the N_e of a population is an important and semi-reliable source to predict survival potential of the population in a changing environment rather than population size (N) (Schwartz *et al.* 2012). However, neither N_e nor N_e/N has been estimated for any pine marten population yet (Buskirk & Ruggiero 1994).

1.4 Method development

A number of different types of DNA genetic markers have been developed and may be applied for various purposes such as to investigate population genetic structure, gene flow, individual identification and phylogeography. The selection of marker particularly depends on which taxonomic level being examined (Alacs *et al.* 2010). Some of the commonly used methods are described below.

Mitochondrial DNA (mtDNA)

Several laboratory methods can be used for species identification from scat and hairs, most of which rely on the amplification of mitochondrial DNA (mtDNA) (Farrell, Roman & Sunquist 2000; Rodgers & Janečka 2013). The mammalian mtDNA comprises roughly 37 genes coding for 22 tRNAs, 2 rRNAs and 13 mRNAs within the cytochrome b coding region. MtDNA polymorphisms are widely used for investigating population structure, interspecies variability and evolutionary relationships between species (Mburu & Hanotte 2005). The D-loop is used for the studies of intraspecific variation while the cytochrome b region is used for interspecies variation (Mburu & Hanotte 2005). In all species, a few short intergenic segment and a large non-coding region located in the gene. The length of the non-coding region varies among species, but the nucleotide sequence exhibits similarities in the central part (Mignotte *et al.* 1990). The large central-conserved sequence block is considered the most conserved region of mtDNA while other parts of the non-coding region are the least conserved sequences of the molecule between species (Mignotte *et al.* 1990). MtDNA has some special characteristics including maternal inheritance, high mutation rate, lack of recombination and a higher copy number than nuclear DNA (Rodgers & Janečka 2013). Short fragments of mtDNA are typically used for the direct sequences (Rodgers & Janečka 2013) and are often favored as a genetic marker over nuclear DNA (nDNA) for species identification of wildlife (Alacs *et al.* 2010).

16S rRNA (16S ribosomal RNA) and 18S rRNA gene

These markers are widely used as the tools for the study of phylogeny, ecology and identification of microbial taxa. The 16S rRNA is used for detection of particular species of

bacteria and the 18S rRNA for microbial-eukaryotes (Blaut *et al.* 2002; Caron *et al.* 2009). The 16S rRNA microbial gene consists of 30S small subunit of prokaryotic ribosomes that contains 21 proteins and 1,500 bases (Blaut *et al.* 2002) and shows 9 highly conserved hyper variable regions (Baker, Smith & Cowan 2003). The 18S rRNA sequences have localized 12 highly variable regions (Magnet *et al.* 2014). The sequence information and variability can be applied for designing oligonucleotide probes to detect various level of bacterial taxonomic hierarchy (Blaut *et al.* 2002).

Single nucleotide polymorphisms (SNPs)

SNPs are an emerging class of molecular markers having a great potential application in wildlife population genetics. A SNP's has a specific site in DNA consisting of 4 possible nucleotides variants. Even a variation in a single nucleotide in SNP site results in formation of different alleles. The alleles identified in a SNP site in an individual can be compared with the alleles of other individuals to examine genetic variation or similarity in individuals (Silvy 2012). SNPs occur throughout the genome in both coding and non-coding regions of DNA in many species and are thought to be well understood their mutation modes. SNP-based genetic can be easily standardized because these data do not depend on the laboratory and technology used. These advantages facilitate uses of SNPs in ecology, evolutionary study and conservation biology (Morin, Luikart & Wayne 2004; Kraus *et al.* 2015). However, the SNP's based markers have some limitations regarding identification of alleles in a particular SNP site, amplification of alleles require more manual effort and consume a large amount of DNA for genotyping (Kraus *et al.* 2015).

Microsatellites

Microsatellites are simple tandem sequence repeats composed of 1- 6 base pair (bp) found at high frequency in the nuclear DNA (Selkoe & Toonen 2006). Microsatellite loci are also known as short tandem repeats (STR), simple sequence repeats (SSR) and variable number of tandem repeats (VNTR). Generally, the length of a microsatellite locus varies in the range between 5-40 repeats but it is also possible to have longer strings of repeats (Selkoe & Toonen 2006; Kalia *et al.* 2011). The variation in microsatellites is mainly due to changes in the number of repeated base pairs (2-6 DNA bases) at a locus that are repeated from 2 to several 100 times. They are most abundant in non-coding regions of the genome where they possess a higher mutation rate than coding regions (Wich 2009; Abdul-Muneer 2014). Among these repeat classes, dinucleotide, trinucleotide and tetranucleotide repeats are the

most commonly used repeats for molecular genetic studies (Selkoe & Toonen 2006). The dinucleotide shows higher amplification success and lowers error rate in compare to long repeat units (Broquet, Ménard & Petit 2007). The dinucleotide repeats –GT and –CA are believed to be the most common microsatellites in vertebrates (Abdul-Muneer 2014). Mononucleotide repeats and longer repeat types are less commonly used nucleotide in genetic study because of problems with amplification (Selkoe & Toonen 2006). Microsatellites are highly polymorphic, co-dominant in nature and randomly distributed throughout the genome. They can easily mutate and are inherited in a Mendelian manner. These properties make the microsatellites ideal genetic markers useful for conservation genetics and advances in population structure demographic analysis (Zane, Bargelloni & Patarnello 2002; Mburu & Hanotte 2005; Kalia *et al.* 2011; Abdul-Muneer 2014). Currently, there are no specific developed microsatellite markers developed for *M. martes*, but it is possible to test cross amplification of loci identified in related species which can be used for European pine marten (Mullins *et al.* 2010). O'Mahony, Turner and O'Reilly (2015) and Balestrieri *et al.* (2016a) studies showed that the microsatellite markers which were tested for other mustelid species successfully amplified genomic DNA of European pine marten.

Aim of the study

The present study is a part of the ongoing BEcoDyn project. The goal of the project is to give insights into the mechanism shaping the dynamics of the ecological community in the boreal forest. This study was initiated to estimate population density of pine marten and gene flow by using microsatellite markers between BEcoDyn project area at Atna and Evenstad and the Fugdallan area close to Rendalen. This is the first attempt to study population genetic of pine marten using microsatellite markers. However, due to limited samples obtained from the field; it was not possible to estimate population density. Also, the success rate of DNA extraction was limited. Therefore, I modified my aim of the study and focus on developing the microsatellite panel (test the specificity of microsatellite markers for PCR amplification and genotyping) useful for determining population genetic structure of Norwegian pine marten. This was conducted by using cross amplification of microsatellites developed for other mustelid species. Here, microsatellite markers were selected from the literature and grouped into 3 multiplex panels based on their suitable combination and optimized panel composition. The suitability of markers was then tested on capillary electrophoresis by using DNA extract from each individual sample. The second aim of my study was to evaluate the methods of DNA extraction from biological samples. In order to achieve this, DNA was isolated from different biological samples (hair follicles, tissues and dry skin with fur) using different methods and tested on PCR amplification and agarose gel electrophoresis.

2. MATERIALS AND METHODS

2.1 *Sample material*

For extraction of DNA, I used hair samples from pine marten traps, muscle tissues from previously caught animals and dry skin sample from the museum collection. The hair samples were collected from non-invasive survey conducted in two sampling sessions, spring /fall in 2014-2015 in 6 systematic plots (each small block~16 km^2 and large block~35km^2 each) within the BEcoDyn project area of Hedmark County. The hair tubes traps were baited with peanut butter/honey. Hair tubes traps were placed on trees at breast height within an approximate spacing interval of ~500 m, which is considered as the average home range of pine marten (O'Mahony 2014). Two sticky glue tape patches were attached to the bottom of each hair tube to allow hair sample collection. The tubes were checked regularly and hair samples from each patch were collected into an Eppendorf tube filled with 96 % ethanol. The samples were stored at -20°C until isolation of DNA. Dry skin with fur samples (museum samples) were collected from the Natural History Museum of Oslo (by courtesy of Øistein Wiig). The fresh muscle tissues samples of killed pine marten with some additional frozen samples obtained from Lista Nature museum (by courtesy of Roar Solheim) and hunters.

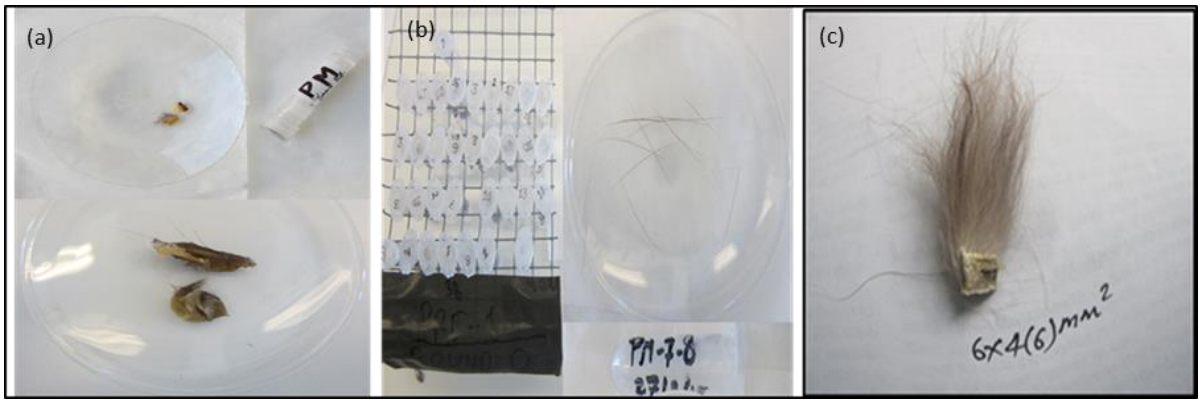


Figure 1: Pictures of 3 types of samples used in this study for the extraction of DNA. **(a)** Fresh tissue samples (meat and skin) of killed pine marten provided by Lista Nature Museum and hunters. **(b)** Hair samples collected from the BEcoDyn area by field workers. **(c)** Skin with fur (museum specimen) collected from Natural History Museum of Oslo.

2.2 DNA extraction

Genomic DNA was extracted from 3 types of samples using various types of methods previously established for biological samples. In my study, I used different methods for extraction of DNA and if one method did not work I tested for another method sequentially. Here methods from Grimsø research station, Lysis buffer and NaOH did not yield target amount of DNA (based on Nano drop measurement) from hair sample in my pilot study (data not shown). Therefore, I selected Chelex 100 protocol and InstaGene Matrix (Chelex resin base) method that yielded a good amount of DNA from hair follicles. These are described in methods section. I aimed to select suitable DNA extraction methods according to sample types such as tissues, hair and skin with fur (museum specimen). The methods that yielded good concentration of DNA were used for the extraction of DNA and samples were further tested in PCR multiplex.

2.2.1 The hair samples

The Chelex method

Isolation of DNA was carried out following the method in Suenaga and Nakamura (2005). Briefly, 5-10 hairs were collected from a single barb. The root segment with follicle (~1cm length) was cut from each hair shaft and collected in a 1.5 mL microcentrifuge tube. The flame-sterilized scissors was used to cut the hair follicles for each pine marten individual sample. 50-100 µL of 5 % Chelex 100 (BIORAD; Richmond, CA, USA) and 5 µL of 20 mg / mL. Proteinase K (OMEGA; bio-tek, USA) was added into the tube containing hair follicles. Then samples were mixed and incubated at 55° C for 1-3 hours (hrs) or alternatively overnight. The mixture was vortexed and incubated at 98^{o} C for 8 minutes (min). After centrifuging at 10,000-15,000 rpm (14.5 x g) for 2-3 min, the supernatant with DNA was transferred to another 1.5 mL microcentrifuge tube. The isolated DNA was stored at 4 °C or -80 °C until further analysis.

The InstaGene Matrix (Chelex based resin)

The InstaGene Matrix (BIORAD; Richmond, CA, USA) kit was also used for extraction of DNA from hair follicles. 5-10 hairs from a single barb for each pine marten sample were collected in1.5 mL microcentrifuge tube. 250 µL InstaGene Matrix was added and spun (10-15 minutes) and incubated at 56 °C for 1hour. The sample was centrifuged at high speed (14,000 rpm) and incubated at 98° C for 15 min. Then, vortexed at high speed for 10 sec followed by centrifuging at 12,000 rpm for 3 min. The supernatant was transferred into a new 1.5 mL microcentrifuge tube and stored at -20°C for further analysis.

2.2.2 The tissue samples

The ISOLATE II Genomic DNA kit (250 preps) method

The ISOLATE II Genomic DNA kit (BIOLINE; London, UK) was used for extraction of genomic DNA from tissue samples. 180 µL Lysis Buffer GL and 25 µL Proteinase K solution was added to approximately 25 mg of small pieces of tissue into a 1.5 mL microcentrifuge tube and incubated at 56 °C for 1-3 hrs until the sample was dissolved. 200 µL of Lysis Buffer G3 was added and incubated at 70 °C after vortex. The supernatant was transferred to an ISOLATE II Genomic DNA spin column in 2 mL collection tube and 210 µL ethanol (96-100 %) was added to adjust DNA binding. 500 µL of Buffer GW1 (Washing Buffer) was added and centrifuged at 11,000 x g for 1 min. Then, 600 µL wash Buffer GW2 was added and centrifuged at 11,000 x g for 1 min. The centrifuging process was repeated once at high speed and incubated at 50 °C for 5 min by opening the lid to remove ethanol. After placing the ISOLATE II Genomic DNA Spin Column in 1.5 mL microcentrifuge tube, 50- 100 µL of preheated Elution Buffer G (70 °C) was added directly onto the silica membrane. After allowing standing at room temperature for 1 minute, the tube was centrifuged at 11,000 x g for 1 minute. The elution containing DNA was collected in 1.5 mL microcentrifuge tube for PCR analysis.

DNA extraction from tissue samples using QIAamp® DNA investigator kit (50)

DNA from tissues was extracted using a protocol provided by the manufacturer (QIAGEN, Germany). 300 µL Buffer ATL, 20 µL proteinase K and 20 µL 1 M DTT were added to approximately 25 mg tissue samples placed in 1.5 mL microcentrifuge tube and incubated at

56 °C for at least 1hr or overnight until the sample was completely dissolved. 300 µL of Buffer AL (Lysis Buffer) was added and incubating at 70 °C for 10 min. Then 150 µL ethanol (96-100 %) was added after vortexed mixture was transferred in a QIAamp MinElute spin column. 500 µL of Buffer AW1 (Washing Buffer) was added and centrifuged at 8,000 rpm for 1 min. The QIAamp spin column was placed in 2 mL collection tube and added 700 µL of Buffer AW2 (Washing Buffer), then centrifuged in high speed at 14,000 rpm for 3 min to dry the membrane. The QIAamp spin column was incubated at 50 °C for 1-2 min to remove ethanol. Same QIAamp spin column was placed in 5 mL microcentrifuge tube and added 20–50 µL of Buffer ATE (Elution Buffer) to resuspend the DNA. The microcentrifuge tube was centrifuged at 14,000 rpm for 1 min. The extracted DNA was collected in 1.5 mL microcentrifuge tube and stored at -20 °C.

2.2.3 The museum samples (dry skin with fur)

DNA extraction from dry skin by using NaOH method

Extraction of DNA and purification process were conducted according to the protocol by Wang, Qi and Cutler (1993). A small piece (about 25 mg) of skin with fur was sliced and added to 100 µL 0.2 M NaOH solution and incubated at 95 °C for 2 hrs or overnight until completely dissolving the sample. The mixture was vortexed and centrifuged for 5 min (14,000 rpm) and the supernatant was transferred to a new microcentrifuge tube. 100 µL 0.1 M Tris-HCl, pH 8.0 was added, vortexed and spun for 1/2 min to neutralize NaOH. The mixture was incubated at - 20 °C for 15-30 min.

DNA clean-up process (Extracted from NaOH method) using Phenol-Chloroform

The purification of isolated DNA from proteins or salts was performed by using following protocol. In brief, equal volume i.e. 100 µL Phenol and 100 µL Chloroform: Isoamyl alcohol (ratio 25:24:1) was added and mixed well. The mixture was vortexed and spun for 5 min to make a homogenous mixture and was left to settle inside a fume hood. The mixture was centrifuged at 11,000 rpm for 15 min. The supernatant (upper clear level) was transformed to a new microcentrifuge tube and sediments (protein and membranes) were discarded. Then double volume of Phenol-Chloroform: Isoamyl alcohol (~300 µL) 96 % ethanol (absolute) and 1/10 th vol. (~30 µL) 3M NaOAC (pH 4.8-5.2) was added to neutralize Phenol-Chloroform and incubated in ice or at -20 °C for 10-30 min. After centrifuged at 14,000 rpm at 4 °C for 15 min supernatant was discarded. 200 µL 70 % ethanol was added and spun at full speed at room temperature for 10 min to wash the pellet. The supernatant was discarded

and the pellet was air dried. The purified DNA Pellet was located and resuspended in 25 µL 0.1 x TE buffers (preheated at 65 °C), then again incubated at 70-90 °C for 5 min and spun to yield high-quality DNA.

DNA clean-up process (DNA extracted by NaOH method) using Wizard Genomic DNA kit

I also tested another DNA clean up protocol developed by Wizard Genomic DNA Purification Kit (www.promega.com/tbs/) for same DNA. I followed the procedures as given by the manufacturer. The cleaned DNA was stored at 4 °C or -20 °C for further analysis.

DNA extraction from dry skin using QIAamp® DNA investigator kit (50)

I isolated DNA from dry skin using a protocol provided by the manufacturer (QIAGEN, Germany) as explain in DNA extraction method from a tissue sample in detail.

2.3 Microsatellite analysis and DNA amplification

2.3.1 Selection of PCR microsatellite markers

Initially, 20 microsatellite loci and one sex identifying marker (K9Amelo) were selected from the literature identified in genomic DNA for other mustelid species being tested for cross-species amplification (Mullins *et al.* 2010; O'Mahony 2014). This microsatellites loci were; Gg7, Ggu234, Gg454, Lut615, Lut604, 040T14, Ma1, Ma2, Ma5, Ma8, Mel1, Mel6, Mel105, MLUT27, Mer041, Mvi1341, Mvi1354, Mvis072, Mvis075, Mvis020 and K9Amelo (Table 1).

2.3.2 Singleplex PCR

All the 21 microsatellite markers were amplified in singleplex (i.e. each marker was run separately in the PCR) with different annealing temperature ranges between 54 °C and 64 °C. I changed the temperature with 2 °C for each PCR reaction to optimize amplification of marker in singleplex. The accuracy of the different melting temperature (TM) of primers was adjusted using the Oligo Analysis software program https://eu.idtdna.com/calc/analyzer, developed by DNA Technologies, Inc. US.

Table 1. Summary of microsatellite loci that were derived from published articles including literature sources and polymorphism characteristics. These microsatellite loci were used for genotyping of pine marten in this study.

Locus	GenBank Assession no.	Primer sequences: 5' to 3' (F = forward, R = reverse)	Fluorescent dye labelled	No. of Alleles	Size Range (bp)	References
Ma-1	AF075137	F: ATTTTATGTGCCTGGGTCTA R: TTATGCGTCTCTGTTTGTCA	FAM	10	206-219	DAVIS and STROBECK-1998
Ma-2	AF075138	F: ACCCATGAATAATGTCTTAT R: ATCTTGCATCAACTAAAAAT	FAM	5	168-180	DAVIS and STROBECK-1998
Ma-8	AF075143	F: GTTTTCTAATGTTTCGTGTG R: CAGTGGTTGACTACAAGAAA	HEX	8	120-130	DAVIS and STROBECK-1998
Gg-7	AF075153	F: GTTTTCAATTTTAGCCGTTCTG R: GTTTATCTCCCTCTTCCTACCC	HEX	6	154-172	DAVIS and STROBECK-1998
Ggu454	AF247757	F: CTTCTTACATAGTCAATGTTTTG R: TGCCATTTTCTCCAGAA	TAMARA	5	127-137	WALKER et al 2001
Mvi 1341	AF480850	F: GTG GGA GAC TGA GAT AGG TCA R: GGC AAC TTG AAT GGA CTA AGA	FAM	12	148-176	VINCENT et al 2003
Mvi 1354	AF480852	F: CCA ACT GGA GCA AGT AAA T R: CAT CTT TGG GAA AGT ATG TTT	FAM	11	166-200	VINCENT et al 2003
Mvis072	AF132104	F: CTGCAAAGCTTAGGAATGGAGA R: CCACTACACTGGAGTTTCAGCA	FAM	4	263-300	FLEMING et al 1999
Mvis075	AF132105	F: GAAATTTGGGGAATGCACTC R: GGCAGGATAGGATGTGAGCT	TAMARA	8	145-180	FLEMING et al 1999
Mvis020	AF132101	F: GGGTCAAGAGTTAGAGCCCC R: GACATGGTCAGATTCCCCAC	TAMARA	7	175-187	FLEMING et al 1999
MLUT27	F093589	F: GCCGAATGTATTAATTACATGG R: GTTTCAGAGGTAATTTGGGAGAC	HEX	2	110-164	CABRIA et al 2007
Mel1	AF300707	F: CTGGGGAAAATGGCTAAACC R: AATGCAGGCTTTGCAATTCC	FAM	3	106-150	BIJALSMA et al 2000
Mel6	AF300712	F: AAGTCCTCCTTGCAGTTTGG R: AGCAAGCTCTTGGTTCTTGG	FAM	2	133-175	BIJALSMA et al 2000
Mel105	AJ293350	F: GATATTCCCCTCCCACCACT R: CTCCAAGGGATCCTGGAACT	FAM	8	136–150	CARPENTER et al 2003
Lut604	Y16295	F: TATGATCCTGGTAGATTAACTTTGTG R: TTCAACAATTCATGCTGGAAC	FAM	5	157-211	DALLAS et al 1998
Lut615	Y16296	F: TGCAAAATTAGGCATTTCATTCC R: ATTCTCTTTTGCCCTTTGCTTC	FAM	7	244-262	DALLAS et al 1998
04OT14	AY786987	F: GGTCCAAGTCCAAGCCTGCCT R: TTCATATTCTTCAGGTGAATCCCAT	HEX	5	108-158	HUANG et al 2005
K9Amelo	NC006621	F: GTGCCAGCTCAGCAGCCCGTGGT R:TCGGAGGCAGAGGTGGCTGTGGC	FAM	5	190-204	LAU et al 1989

2.3.3 Multiplex PCR

After selecting the microsatellites and testing in singleplex PCR reaction, microsatellites multiplex PCR was developed for simultaneous amplification of multiple loci in the same reaction. The markers concentrations were further modified based on agarose gel electrophoresis result and PCR amplification of unlabeled primers. The fluorescent dyes labeled primers sets were grouped into their corresponding multiplexes depending on their colors and sizes without overlapping using the software multiplex manager (version 1.2) found at http://multiplexmanager.com . The reaction conditions, annealing temperature, volume and final concentration of markers and concentration of $MgCl_2$ were adjusted for the multiplex. Three microsatellite markers (Ma5, Ggu234 and Mer041) did not amplify in to PCR multiplex; therefore they were removed from the multiplex. Finally, 17 microsatellite loci and 1 sex identifying markers were used for the multiplex PCR. Identification of sex is important when studying elusive carnivores and other species. The amelogenin (AMEL) is a conserved gene localized on the sex chromosomes of mammals which were used as a marker to identify sex (Lau *et al.* 1989). The marker K9Amelo was included in the multiplex PCR. Multiplex 1 contained the following six markers Ggu454, MLUT27, Lut604, Mel105, Ma2 and Mvi1354. Similarly, Multiplex 2 contained eight primers which were Mvis075, Ma8, Gg7, Mel1, Mvi1341, Ma1, Lut615 and Mvis072, while Multiplex 3 contained three primers and one sex identifying marker; Mvis020, 04OT14, Mel6 and K9Amelo (Figure 2).The multiplex panels of 4 to 8 different markers were genotyped together in a 16 capillary 3130xl Genetic Analyzer (Applied Biosystems).

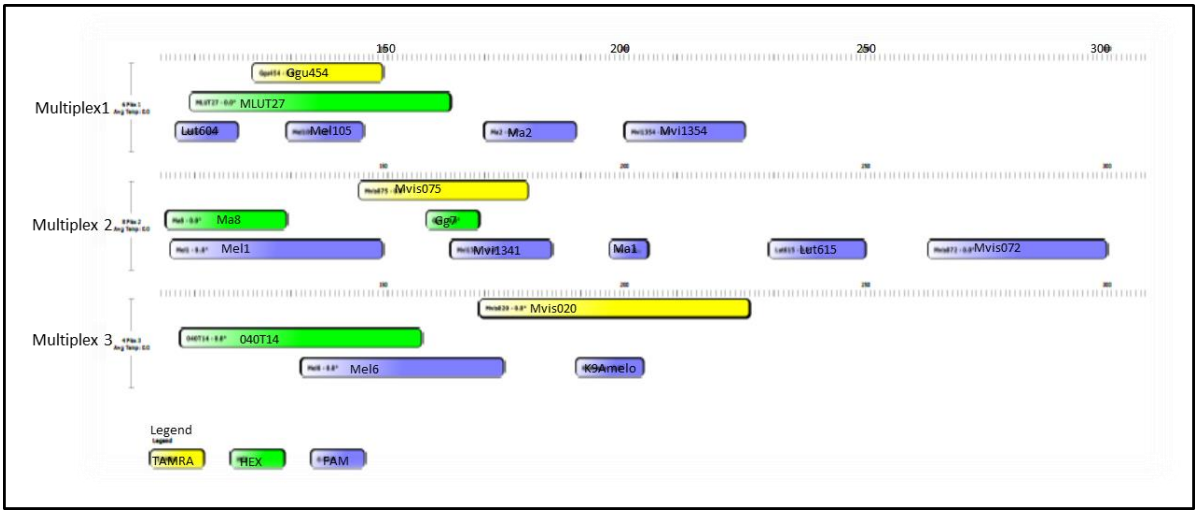


Figure 2. **Pine marten YSAT multiplex panels (4 base pair)**. Multiplex 1 consists of 6 markers namely Ggu454, MLUT27, Lut604, Mel105, Ma2 and Mvi1354. Multiplex 2 contains 8 markers such as Mvis075, Ma8, Gg7, Mel1, Mvi1341, Ma1, Lut615, and Mvis072. Multiplex 3 lies 4 markers Mvis020, 040T14, Mel6 and K9Amelo. Multiplex panel was generated by the software multiplex manager (version 1.2) found in *http://multiplexmanager.com*.

2.3.4 PCR amplification

The selected microsatellite markers were tested using a variety of PCR reactions on DNA extracted from fresh tissue and hair samples. Initially, PCR amplification was performed in a total volume of 15 µL. The reaction mixture contained 1.5 µL 10 x B2 reaction buffer (10 µM), 0.9 µL (25 µM) MgCl2, 0.15 µL (10 µM) deoxynucleotide triphosphates (dNTPs) (BWR), 0.15 µL (10 mg /µL) bovine serum albumin (BSA), 0.1 µM of forward primer and reverse primer, 0.15 µL (5 U/µL) Hot fire polymerase and 1µL of DNA template. PCR water was added in to the primer set of singleplex and multiplex to adjust total PCR reaction volume.The PCR program was 95 °C initial denaturation for 10 min, followed by 35 cycles of 95 °C for 15 sec, annealing at 54-64 °C for 15 sec, extension at 72 °C for 45 sec, later followed by a single final extension time at 72 °C for 7 min pausing at 10 °C. The PCR program was slightly modified until all the markers were amplified in the multiplex panel. This was a vast and time consuming optimization step.

In the meantime, I tested another PCR reaction aiming to reduce the cost associated with multiplex primers developed by Blacket *et al.* (2012). In the same PCR conditions stated above, I used 0.1 µM forward tailed primer, 0.2 µM reverse primer and 0.1 µM fluorescently tagged universal primer corresponding to each tail primer instead of 0.2 µM forward and

reverse primers. However, this PCR reaction requires more labour and consumes a lot of time for separate optimization of singleplex PCR and multiplex PCR.

2.4 Gel electrophoresis and capillary electrophoresis

2.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to observe amplification of the DNA in the PCR reaction. Here, 5 µL of the PCR product was run in electrophoresis in 2 % (1× TAE) agarose gel (LIFE SCIENCE). 2 % agarose gel was prepared in 72 mL of 1 X TAE buffer by weighing 1.44 g (2 % of TAE Buffer) of agarose powder. The suspension was melted in the microwave until no floating particles could be observed. 1.5 µL of 6 x LB (Loading Buffer) dye was added to PCR amplified samples prior to loading on the gel and visualized by ethidium bromide stain (2 mg / mL). A molecular size marker included in all gels was a 100 bp DNA ladder (BIOLAB). Electrophoresis was performed for 45 minutes at constant voltage (90 V). The gel membrane was examined by UV- transillumination and photographed.

2.5 Capillary electrophoresis

The amplified PCR product was further used for capillary electrophoresis in 16 capillary 3130xl Genetic Analyzer (Applied Biosystems). Finally, all genotyping were scored against a GS500 ROX (TM) (400 µL) Red DNA Size Standard using GeneMapper software (version 4.0) (Applied Biosystems).

3. Results

3.1 DNA extration and evaluation

DNA was successfully extracted from 7 tissue samples, 26 dry skin samples (museum samples) and 49 hair samples of an individual pine marten. The extracted DNA concentration was measured in a Nano drop spectrophotometer for further analysis. The concentration of DNA was varied according to sample types and extraction methods. The DNA isolated from tissue samples using the Isolate-II Genomic Kit varies between 20.2-282.5 ng /µL, while the QIAmp DNA investigator kit (50) yielded 30.1-93.8 ng /µL DNA (Table 2). The fresh tissue samples yielded better quality of DNA than that of frozen tissue samples.

The hair samples yielded 21.3-175.2 ng/µL DNA using the Chelex 100 protocol. When using the InstGene matrix method, less than 6 ng/µL DNA was extracted (Table 3). I found the DNA extracted from hair follicles using Chelex 100 protocol was very unstable and degraded within a week when stored at 4 ° C. Same DNA preserved at -80 ° C under thawing and freezing (-80 ° C) condition remained useful for PCR amplification until 1 month.

Whereas, dry skin samples yielded 10.1-208.5 ng /µL of DNA using the Isolate-II Genomic DNA Kit (50), 4.7-198.9 ng /µL DNA extracted by the NaOH method, while less than 5 ng /µL DNA was extracted using the QIAamp DNA investigator Kit (50) (Table 4).

Samples were selected for the PCR amplification based on concentration, absorbance values of 260/280 and 260/230. The absorbance value of ~1.8-2.0 (ratio of 260/280) was generally accepted as pure for DNA. The DNA having absorbance values less than 1.8 were considered presence of protein, phenol or other contaminants in the DNA (Wilfinger, Mackey & Chomczynski 1997). All the samples having DNA concentration close to 20 ng /µL and absorbance values nearby 1.8 were tested in PCR reaction.

Table 2. **Table showing DNA concentration measured in Nano drop spectrophotometer for tissue samples**. ng/µL indicates the DNA concentration of each tissue sample. Absorption values 260 / 280 and 260 / 230 indicate the purity of nucleic acid.Absorption values of 260/280 ranges between 1.8 – 2.0 and values of 260/230 between 2.0 and 2.2 considered the purity of nucleic acids (DNA and RNA).

Nano drop measurement of DNA isolated from tissue samples						
SN	Sample Code	Sample ID	ng/µl	260/230	260/280	Method
1	PM-NS	PMT1	93.8	2.19	1.89	QIAamp
2	PM-NS	PMT1	30.1	1.04	1.97	QIAamp
3	PM-NS	PMT1	49.5	1.62	1.93	QIAamp
4	PMSI (Same ind)	PMT2	48.2	1.22	1.93	QIAamp
5	PM-NS	PMT1	124.5	2.34	1.95	G.Kit
6	PM-NS	PMT1	94.4	2.18	1.91	G.Kit
7	PMSI (Same ind)	PMT2	282.5	2.5	1.64	G.Kit
8	PMSI (Same ind)	PMT2	22.8	2.74	2.09	G.Kit
9	PML2 (Lista 2)	PMT3	45	0.35	1.76	G.Kit
10	PM3L3 (Lista 3)	PMT4	82	2.12	1.66	G.Kit
11	PM3L3 (Lista 3)	PMT4	123.9	1.72	1.89	G.Kit
12	PML1 (Lista 1)	PMT5	53.3	1.86	1.88	G.Kit
13	PM2L4 (Lista 4)	PMT6	20.2	0.43	1.49	G.Kit
14	PMO1 (Pine Marten1)	PMT7	132	1.62	1.87	G.Kit

Table 3.**Table showing Nano drop measurement of DNA isolated from hair samples.** ng /µL indicates the DNA concentration of each extraction. Absorption values 260 / 280 and 260 / 230 indicate the purity of nucleic acid. Absorption values of 260/280 between 1.8 – 2.0 and 260/230 between 2.0-2.2 considered the purity of nucleic acids (DNA and RNA). The samples masked with gray colour (Italic) indicate amplified DNA. Last column showed the number of hair follicles used in DNA extraction.

Nano drop measurement of DNA isolated from hair follicles (HF) (BEcoDyn area)							
SN	Trap Code	Sample ID	ng/µl	260/230	260/280	Method	No. of HF used
1	PM-3-4	PMH1	3	0.36	2.16	InsGeneM	5 HF

Nano drop measurement of DNA isolated from hair follicles (HF) (BEcoDyn area)							
SN	Trap Code	Sample ID	ng/µl	260/230	260/280	Method	No. of HF used
2	PM-5-1	PMH2	3.9	0.49	1.3	InsGeneM	5 HF
3	PM-5-10	PMH3	5.3	0.42	1.92	InsGeneM	5 HF
4	PM3-25	PMH4	5.6	0.36	1.78	InsGeneM	10 HF
5	PM-1-5	PMH5	21.3	0.25	1.95	Chelex	1 HF
6	PM-2-7	PMH6	22.2	0.25	1.74	Chelex	2 HF
7	PM-7-6	PMH7	80.5	0.17	0.62	Chelex	4 HF
8	PM-3-29	PMH8	79.3	0.17	0.62	Chelex	4 HF
9	*PM-6-3*	*PMH9*	*103.5*	*0.93*	*1.73*	*Chelex*	*5 HF*
10	*PM-4-6*	*PMH10*	*30.8*	*0.41*	*1.14*	*Chelex*	*5 HF*
11	*PM-1-8*	*PMH11*	*31.4*	*0.21*	*1.01*	*Chelex*	*5 HF*
12	PM-1-9	PMH12	42	0.15	0.66	Chelex	5 HF
13	PM-3-16	PMH13	47	0.16	0.67	Chelex	5 HF
14	PM-4-1	PMH14	75.1	0.17	0.62	Chelex	5 HF
15	PM-7-7	PMH15	82.4	0.18	0.63	Chelex	5 HF
16	PM-1-3	PMH16	55.9	0.15	0.63	Chelex	6 HF
17	PM-3-9	PMH17	40.3	0.15	0.63	Chelex	6 HF
18	PM-3-25	PMH18	57.3	0.16	0.61	Chelex	6 HF
19	PM-1-9	PMH19	43	0.15	0.63	Chelex	6 HF
20	PM-1-8	PMH20	49	0.15	0.62	Chelex	6 HF
21	PM-7-13	PMH21	50.2	0.16	0.64	Chelex	7 HF
22	PM-3-10	PMH22	44.4	0.15	0.65	Chelex	7 HF
23	PM-3-12	PMH23	53.1	0.16	0.63	Chelex	7 HF
24	PM-1-3	PMH24	40.5	0.15	0.64	Chelex	8 HF
25	PM-3-1	PMH25	51.6	0.16	0.64	Chelex	8 HF
26	PM-7-21	PMH26	54.3	0.16	0.65	Chelex	8 HF
27	PM-1-8	PMH27	45.4	0.16	0.66	Chelex	9 HF
28	PM-4-6	PMH28	42.7	0.15	0.63	Chelex	9 HF
29	PM-4-91	PMH29	39.6	0.15	0.65	Chelex	9 HF
30	PM-3-14	PMH30	24.5	0.15	0.66	Chelex	10 HF
31	PM-1-1	PMH31	22.6	0.15	0.68	Chelex	10 HF
32	PM-6-20	PMH32	24.4	0.14	0.65	Chelex	10 HF
33	PM-1-3	PMH33	21.5	0.15	0.7	Chelex	10 HF
34	PM-4-91	PMH34	175.2	0.56	1.11	Chelex	10 HF
35	*PM-4-8*	*PMH35*	*29.7*	*0.19*	*0.85*	*Chelex*	*10 HF*
36	*PM-4-8*	*PMH36*	*36*	*0.19*	*0.82*	*Chelex*	*10 HF*
37	*PM-3-4*	*PMH37*	*51.6*	*0.22*	*1.01*	*Chelex*	*10 HF*
38	*PM-1-15*	*PMH38*	*50.5*	*0.21*	*0.94*	*Chelex*	*10 HF*
39	*PM-4-4*	*PMH39*	*42.3*	*0.2*	*0.94*	*Chelex*	*10 HF*
40	PM-1-9	PMH40	120.7	0.86	1.28	Chelex	10HF
41	PM-1-8	PMH41	47.2	0.22	1.01	Chelex	10 HF
42	PM-4-91	PMH42	175.2	0.56	1.11	Chelex	10 HF

Nano drop measurement of DNA isolated from hair follicles (HF) (BEcoDyn area)							
SN	Trap Code	Sample ID	ng/µl	260/230	260/280	Method	No. of HF used
43	PM-4-8	PMH43	29.7	0.19	0.85	Chelex	10 HF
44	PM-4-8	PMH44	36	0.19	0.82	Chelex	10 HF
45	PM-3-4	PMH45	51.6	0.22	1.01	Chelex	10 HF
46	PM-1-15	PMH46	50.5	0.21	0.94	Chelex	10 HF
47	PM-4-4	PMH47	42.3	0.2	0.94	Chelex	10 HF
48	PM-1-9	PMH48	120.7	0.86	1.28	Chelex	10 HF
49	PM-1-8	PMH49	47.2	0.22	1.01	Chelex	10 HF

Table 4. Nano drop measurement detail of DNA isolated from dry skin samples (museum samples). ng/µL indicates the DNA concentration of each sample. Absorption values 260/280 and 260/230 indicate the purity of nucleic acid. Absorption values of 260/280 ranges between 1.8 – 2.0 and 260/230 ranges between 2.0-2.2 are considered the purity of nucleic acids (DNA and RNA).

Nano drop measurement of DNA concentration isolated from dry skin (museum sample)						
SN	Sample Code	Sample ID	ng/µl	260/230	260/280	Method
1	PM-14131	PMD1	198.9	1.44	1.82	NaOH
2	PM-11849	PMD2	117.6	1.99	1.64	NaOH
3	PM-7/95	PMD3	42.7	0.37	1.29	NaOH
4	PM-151	PMD4	43	1.11	1.47	NaOH
5	PM-14134	PMD5	48.3	1.79	1.6	NaOH
6	PM-4-82	PMD6	12.8	1.47	1.84	NaOH
7	PM-6/95	PMD7	53.3	0.5	1.39	NaOH
8	PM-14132	PMD8	13.1	0.44	1.59	NaOH
9	PM-11421	PMD9	58.8	0.35	1.37	NaOH
10	PM-11423	PMD10	4.7	0.96	1.44	NaOH
11	PM-1989-18	PMD11	14.4	0.93	1.42	NaOH
12	PM-11365	PMD12	63	0.23	1.51	NaOH
13	PM-14174	PMD13	13.3	1.02	1.58	NaOH
14	PM-5/95	PMD14	83.8	0.27	1.12	NaOH
15	PM-14573	PMD15	120.3	1.71	1.75	NaOH
16	PM-14173	PMD16	208.5	2.23	2.06	G.Kit
17	PM-476/68	PMD17	149.7	1.88	1.74	G.Kit
18	PM-14137	PMD18	22.2	1.64	2.01	G.Kit
19	PM-14578	PMD19	12.2	0.62	2.34	G.Kit
20	PM-10933	PMD20	10.1	0.89	2.01	G.Kit
21	PM-476/68	PMD21	193.4	0.73	1.26	G.Kit
22	PM-338/68	PMD22	3.6	0.33	1.32	QIAamp
23	PM-476/68	PMD23	2.3	0.15	1.1	QIAamp
24	PM-1989-17	PMD24	1.9	0.25	1.62	QIAamp
25	PM-338/68	PMD25	2.4	0.35	2.26	QIAamp
26	PM-476/68	PMD26	4.9	0.23	0.62	QIAamp

3.2 PCR amplification

PCR program of the selected microsatellites for the Norwegian pine marten DNA amplification was established for singleplex reactions and multiplex panels. The developed PCR program was tested by using DNA extracted from fresh tissue, hair and dry skin samples. The test result revealed that the PCR program developed in this study can successfully amplify all the microsatellite markers with their respective alleles. Brifly, PCR reaction volume of 15 µL contained 1.5 µL 10 x B2 reaction buffer (10 µM), 1.2 µL (25 µM) MgCl2, 0.15 µL (10 µM) deoxynucleotide triphosphates (dNTPs) (BWR), 0.15 µL (10 mg /µL) bovine serum albumin (BSA), 0.2 µM of each primer, 0.15 µL (5 U/µL) Hot fire polymerase and 1µL of DNA template. PCR water was added to adjust the volume of primer sets singleplex / multiplex in a total reaction volume.The PCR protocol was followed as: 95 °C initial denaturation for 10 min, followed by 35 cycles of 95 °C for 15 sec, annealing at 54 °C for 15 sec, extension at 72 °C for 45 sec, later followed by a single final extension time at 72 °C for 7 min pausing at 10 °C.

The fresh tissue sample successfully amplified of all markers in singleplex reactions and multiplex panels on testing PCR amplification using fluorescently tagged universal primers corresponding to each universal tail primer. Wherease, DNA extracted from hair and museum samples did not amplify the markers.

3.3 Microsatellite markers amplification

1µL DNA template from each individual was used for PCR amplification. The agarose gel electrophoresis was performed to check whether each marker can amplify pine marten DNA in the different PCR conditions. Most of the DNA extracted from the tissues revealed successful amplification for microsatellite primers, yielding DNA fragments of 100-350 base pair (bp) size. The sex identifying marker K9Amelo tested on DNA of same individual tissue sample at three different annealing temperature (54 °C, 56 °C and 58 °C) showed successful amplification (Figure 3 b, lane 4, 7 and 10) producing the strong band of 200 bp. However, only a few DNA isolated from hair samples amplified in PCR reaction, while DNA extracted from dry skin did not amplify the microsatellite markers (Figure 3 b, lanes 2, 3, 5, 6, 8, 9). The size of DNA was observed in an agarose gel electrophoresis picture (Figure 33). The successful amplification of microsatellite sets was confirmed by the presence of bands on a

gel electrophoresis picture. The corresponding size range of the DNA fragment separated on the agarose gel was compared to100 bp DNA ladder (BIOLAB) size standard added in the first left column (Figure 3 a; lane 1 and 18, Figure 3 b; lane 1). A series of experiments were conducted to set final concentration of microsatellite markers and PCR reaction mixture to produce successful amplification of microsatellites in natural DNA. According to the result of many trials, annealing temperatures was modified and set at 54 °C for all primers and sample types.

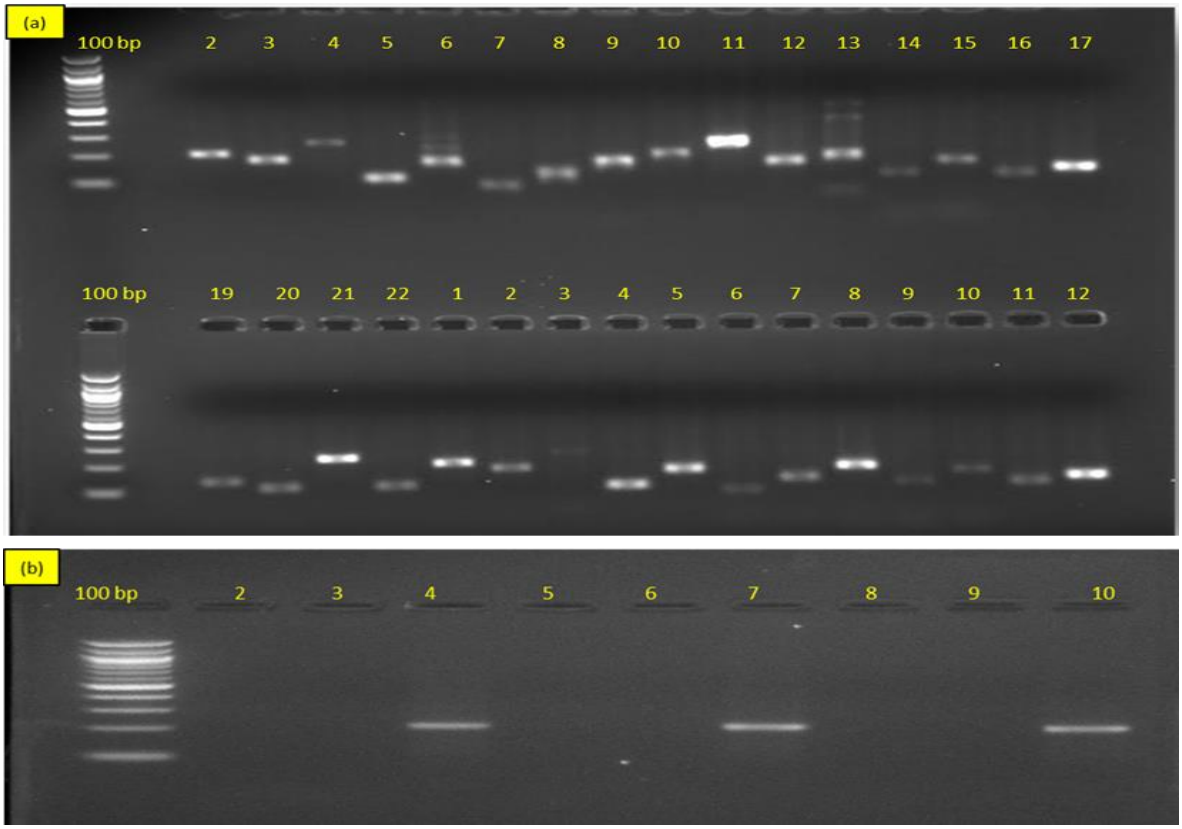


Figure 3. Agarose gel images showing result of PCR amplification of the genomic DNA (tissue sample PMT1). (a) In lane: 1:100 bp DNA ladder (BIOLAB), in rest of the lanes 2µM forward and reverse primers were used, such as: lane 2: Ma1, 3: Ma2, 4: Ma5, 5: Ma8, 6: Gg7, 7:Ggu23, 8: Ggu454, 9: Mvi1341, 10:Mvi1354, 11: Mvis072, 12: Mvis075 , 13: Mvis020, 14: MLUT27, 15: Mer041, 16: Mel1, 17:Mel6, **(a)** Second raw lane 18: 100 bp DNA ladder (BIOLAB), 19:Mel105, 20: Lut604, 21: Lut615, 22:040T14**. (a)** Second raw lane 1: Ma1, 2: Ma2, 3: Ma5, 4: Ma8, 5: Gg7, 6: Ggu234, 7: Ggu454, 8: Mvi1341, 9: Mvi1354, 10: Mvis072, 11: Mvis075, 12: Mvis020. PCR annealing temperatures were 54 °C (for 2-22 primers) and 56 °C (for 1-12 primers). **(b)** Lane 1: 100 bp DNA ladder (BIOLAB), lane 4,7 and 10 sex identifying marker K9Amelo amplified on DNA isolated from same tissue sample. PCR annealing temperatures for sex identifying marker were 54 °C, 56 °C and 58 °C.

3.4 Development of microsatellite panels

Multiplexes of microsatellite markers

PCR products were genotyped on genomic DNA extracted from tissue and hair samples. The microsatellite marker concentration was adjusted based on the intensity of fluorescence signal produced by the microsatellite markers in Gene-Mapper (Figure 4). In figure 4 illustrates the signal intensity given by the four microsatellite markers such as Lut604 (0.75 µM), Mel105 (0.1 µM), Ma2 (0.09 µM) and Mvi1354 (0.15 µM) in multiplex 1 with respect to their corresponding loci on the tissue sample. Every allele was framed into 2-4 bp based on dinucleotide repeats loci which were known as bins. Figure 4 a, was the result visualized from capillary electrophoresis of multiplex set 1 before creating the bins. In such case, bins should be created to standardize the locus sizes within alleles and range of alleles.

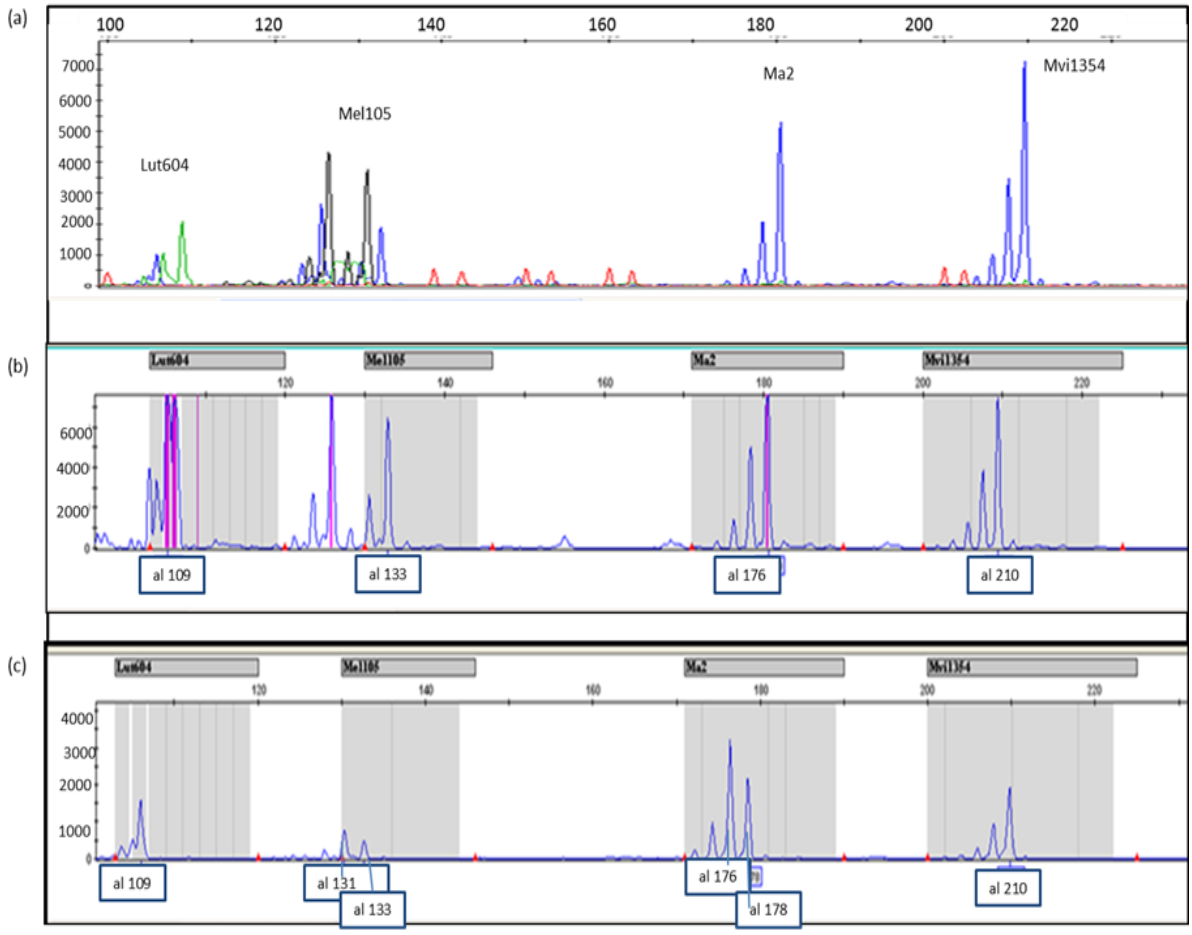


Figure 4. Representative panel diagrams illustrate the process of adjustment of four markers of multiplex set 1 with corresponding loci for tissue sample. **(a)** Panel with the signal of alleles at their corresponding loci visualized before creating bins, giving very high signal intensity around 7,000 units and very low signal around 500 units. **(b)** Panel shows improved signal after increasing or decreasing markers concentration, still one allele was out of range of locus size. **(c)** Panel illustrating

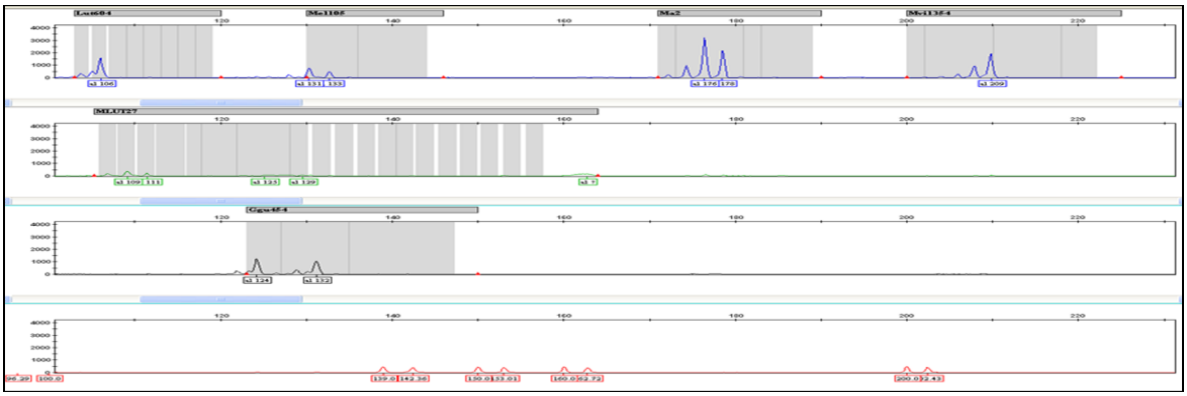
the better signal intensity around 1,000- 3,000 units after the alteration of the markers concentration. The gray area represented the range of each locus size.

The microsatellite markers Lut604 and Mel105 gave low signals whereas Ma2 and Mvi1354 produced very high signals (Figure 4 a). The concentration of markers Lut604 and Mel105 were 0.05 µM to 0.1 µM and 0.09 µM to 0.75 µM respectively. Similarly, primers concentrations of markers Ma2 and Mvi1354 were decreased from 0.15- 0.09 µM and 0.2 - 0.15 µM that produced figure 4 b on genotyping after adjustment of markers concentration. The pink color lines may be due to the effect of color overlapping of other primers in the panel (color push up). The small red color peaks between each allele are the signal of Rox (400 µL) red DNA size standard. In figure 4 c, markers gave the better signal with their respective alleles after final alteration of the markers concentration such as Lut604 (0.1 µM), Mel105 (0.08 µM), Ma2 (0.15 µM) and Mvi1354 (0.20 µM).

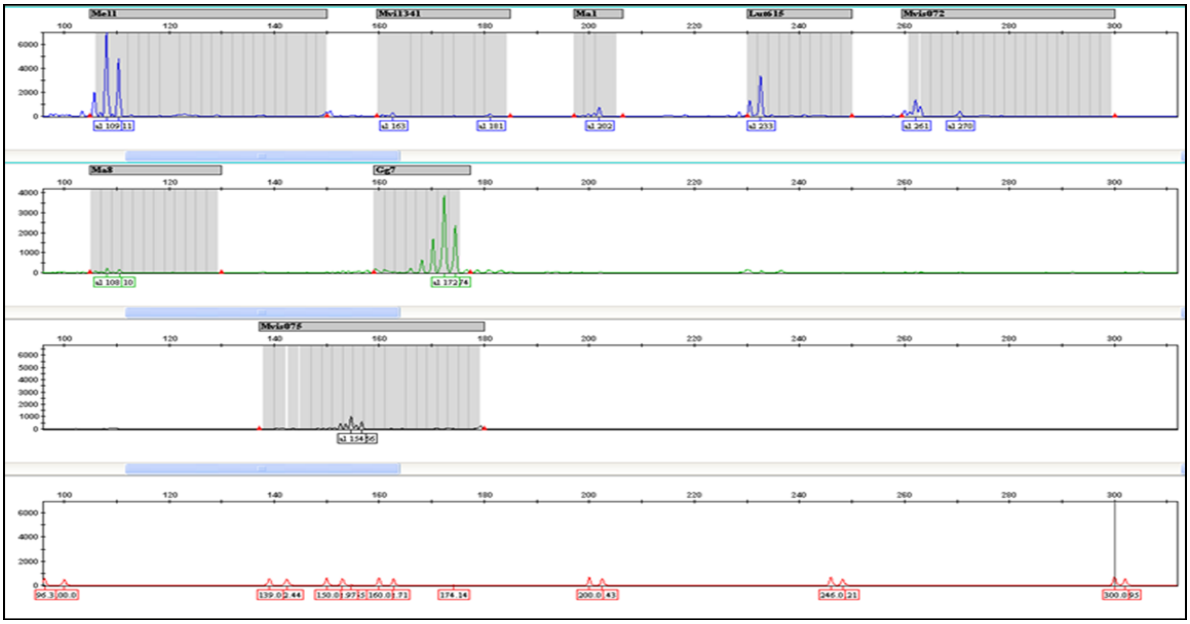
Genotyping success

The genotyping success rate of microsatellite markers was tested by capillary electrophoresis. DNA extracted from 7 tissue samples of each individual was successfully genotyped. Whereas, the DNA isolated from 8 samples were partially successful in genotyping. The alleles for each microsatellite markers for each individual were identified and analyzed (Figure 5 a. for multiplex set 1, Figure 5 b. for multiplex set 2 and Figure 5 c. for multiplex set 3).

(a)



(b)



(c)

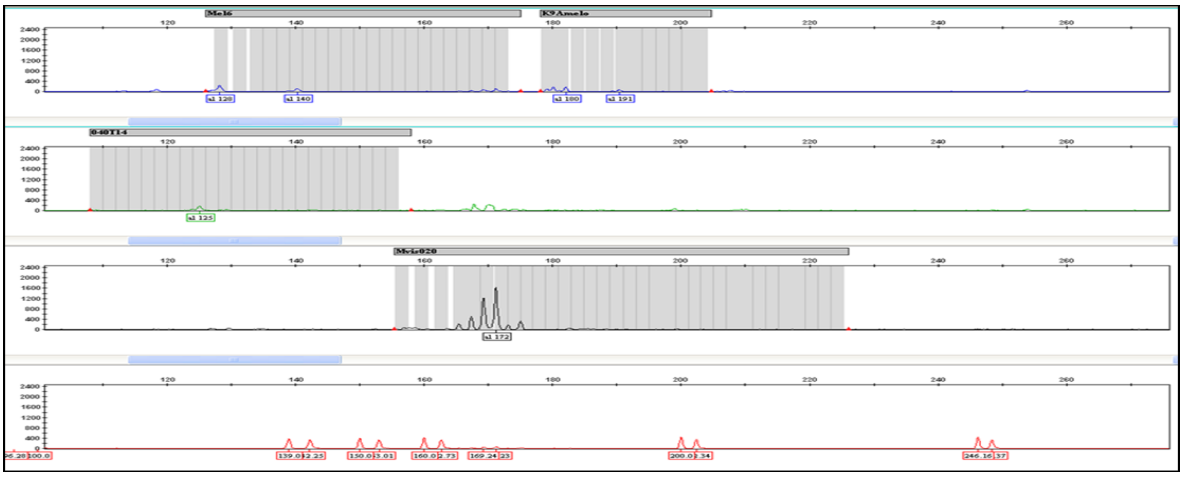


Figure 5. Representative diagrams of microsatellite panels. Each multiplex set has 3 panels having fluorescence labeled markers such as Fam (blue), Hex (green) and Tamra (yellow). **(a)** Multiplex set 1, panel illustrating the sizes of alleles of each marker at their respective loci**. (b)** Multiplex set 2, panel showing the alleles sizes of each marker to their corresponding loci. **(c)** Multiplex set 3, panel highlighted the respective allele by the fluorescence dyes labeled markers. Red color peaks in each multiplex set represented rox (400 µL) size standard and gray areas represented the size range of each locus.

Each multiplex set consists of 3 panels, each panel represented the fluorescence labeled markers such as top panel denoted FAM (Blue), the second panel referred HEX (Green) and last panel for TAMRA (Yellow). The markers labeled with TAMRA should produce yellow color peaks; however, usually, they appeared in black color. This might be due to the result of cross detection of colors overlapping emission curves.

Genotyping result revealed that DNA isolated from fresh tissues sample (PMT1) successfully amplified 100 % microsatellite markers (Table 5). Altogether, 7 tissue samples amplified 79 % microsatellite markers (out of total 18 microsatellite markers) to their respective alleles, while 8 individual hair samples amplified only 32 % of microsatellite markers (Figure 6). However, one of the hair sample (PMH9) DNA amplified 88.7 % microsatellite markers tested on genotyping (Table 5).

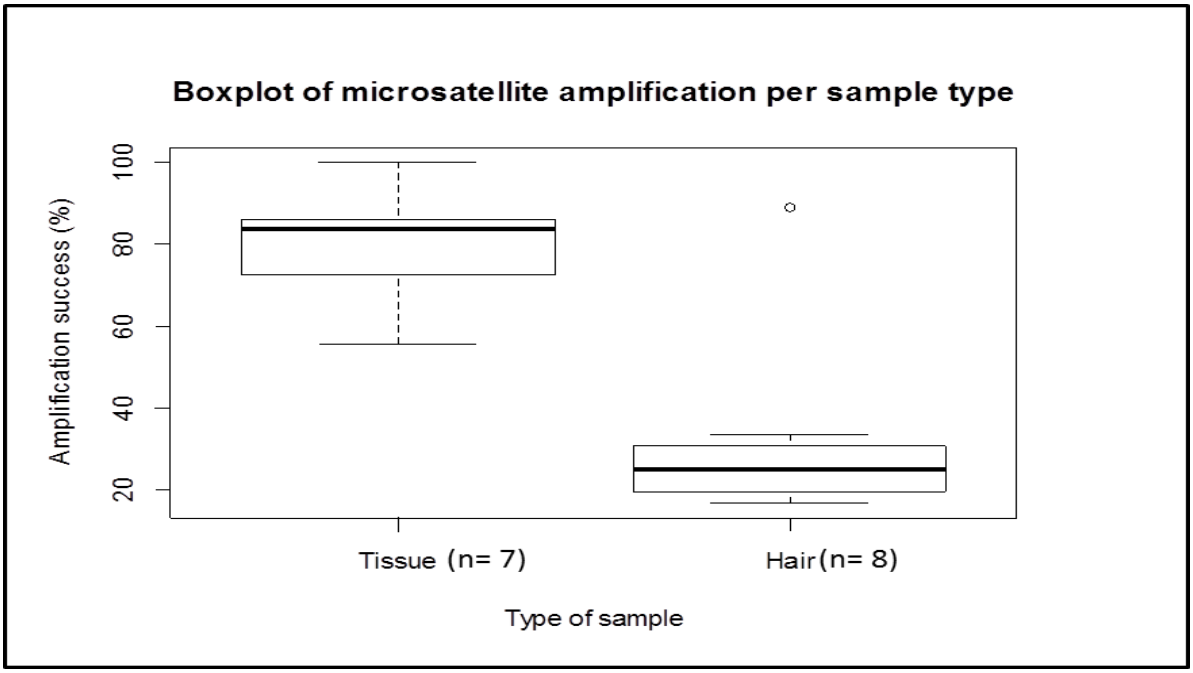


Figure 6. Box plot showing the percentage of successful amplification of microsatellite markers out of 18 microsatellite markers used in this study. Genomic DNA isolated from 7 tissue samples and 8 individual hair samples, fully or partially amplified microsatellite markers on genotyping. Thick black line in the middle of boxplots represents medians. The error bar indicated ± SE.

The markers in each microsatellite panel successfully amplified their corresponding loci according to their size range and fluorescence label dyes tested on fresh tissue sample. The characteristics of 18 microsatellites loci for pine marten from Norwegian population with total alleles were obtained. Out of 18 microsatellite loci, 2 microsatellite loci presented as the examples how the principle of binning carried out in practice (Figure 7 and 8). For instant, the right side of Figure 6 b, 5 bars (n=11; where, n= number of alleles count) were closer to 149 (range between 148.5-149.4 bp); therefore, they were considered as a single allele having allele size 149 bp. Similarly, all the microsatellite loci amplified their respective alleles that showed considerable variation between 2 to 11 alleles per locus (Table 5).

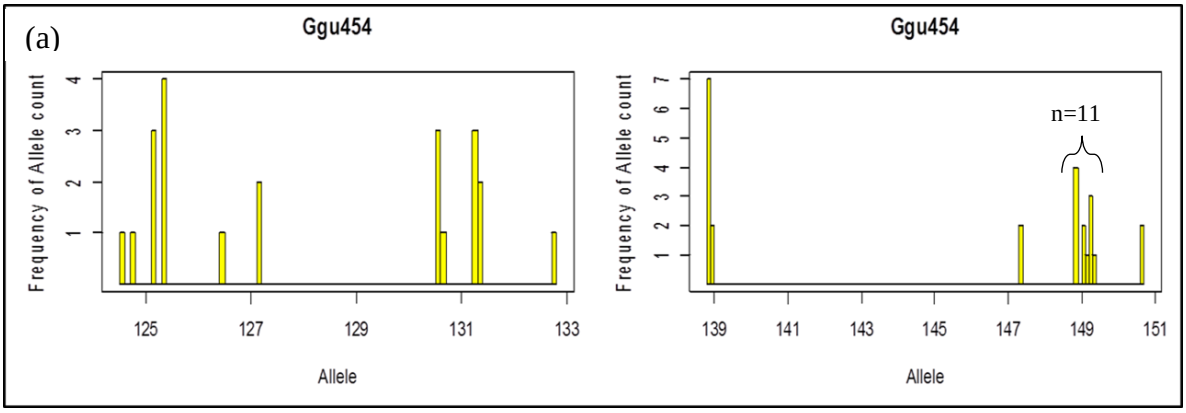


Figure 7. A representative diagram of microsatellite locus with corresponding amplified alleles in its size range. (a) Microsatellite locus Ggu454, alleles range from 125 to 133 base pair (bp). **(b)** Microsatellite locus Ggu454, alleles ranges from 139 to 151 bp. In figure 7 b, 5 bars (n=11) nearby 149 bp were considered a single allele that is 149 bp. This showed the principle of binning of alleles (based on dinucleotide). Yellow colour represents fluorescence dye of locus Ggu454. Where, n= number of alleles count.

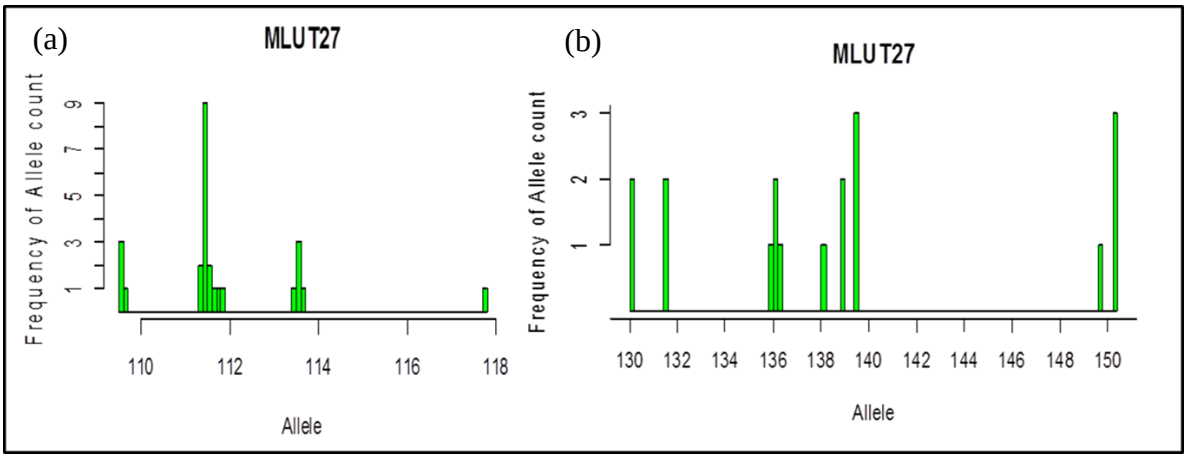


Figure 8. A representative diagram of microsatellite locus with corresponding amplified alleles in its size range. **(a)** Microsatellite locus MLUT27, alleles ranges from 110 to 118 bp **(b)** Microsatellite locus MLUT27, alleles ranges between 130 to 150 bp. Green colour represents the fluorescence dye of locus MLUT27.

Table 5. Characteristics of microsatellite markers with their respective alleles obtained from genotyping. Heading on the top row indicates the names of microsatellite loci, A1/A2 represents alleles and numbers just below it represents the bins (size of alleles) of individual tissue and hair samples. Left first column represents ID of tissue samples and hair samples. Last raw represented the total number of alleles present in each locus. The number at the bottom row represents total number of alleles.

SAMPLE ID	Ggu454	MLUT27	Lut604	Mel105	Ma2	Mvi1354	Mvis075	Ma8	Gg7	Mel1	Mvi1341	Ma1	Lut615	Mvi072	Mvis020	040T14	Mel6	K9Amelo
TISSUE	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2	A1 / A2
PMT1	139 / 149	112 / 114	109 / 109	131 / 133	176 / 178	210 / 210	157 / 161	105 / 105	164 / 170	110 / 118	172 / 172	204 / 206	232 / 236	263 / 265	172 / 188	126 /140	129 / 171	180 / 184
PMT2	125 / 131	112 / 114	x	131 / 133	176 / 178	x	157 / 161	105 / 105	164 / 170	110 / 116	160 / 162	204 / 206	232 / 232	x	160 / 200	126 / 140	129 / 171	180 / 184
PMT3	139 / 147	136 / 150	x	x	176 / 178	x	149 / 159	x	x	x	184 / 184	x	x	263 / 265	172 / 200	136 / 136	129 / 133	180 / 180
PMT4	139 / 151	112 / 130	x	x	180 / 180	x	155 / 157	105 / 111	172 / 174	108 / 112	164 / 182	200 / 202	232 / 232	263 / 271	160 / 200	126 / 136	x	180 / 190
PMT5	139 / 149	112 / 132	109 / 109	131 / 131	180 / 180	x	155 / 157	105 / 113	164 / 168	112 / 118	164 / 164	202 / 204	232 / 232	261 / 263	200 / 200	136 / 136	x	x
PMT6	131 / 131	112 / 118	109 / 109	131 / 137	176 / 180	200 / 200	139 / 141	105 / 117	168 / 172	118 / 120	x	202 / 202	x	261 / 267	x	x	x	x
PMT7	125 / 131	112 / 136	109 / 109	131 / 133	176 / 178	210 / 210	157 / 161	x	164 / 168	110 / 116	172 / 184	204 / 206	x	263 / 271	172 / 188	126 / 140	129 / 185	180 / 184
HAIR																		
PMH9	125 / 125	110 / 112	107 / 107	131 / 133	176 / 178	208 / 110	141 / 141	x	168 / 170	108 / 110	164 / 164	x	132 / 132	171 / 175	172 / 182	126 / 140	129 / 141	180 / 180
PMH10	149 / 149	112 / 140	x	x	x	x	149 / 159	x	x	x	x	x	x	x	160 / 200	x	x	x
PMH11	149 / 149	112 / 140	107 / 107	133 / 133	178 / 180	x	149 / 159	x	x	x	x	x	x	x	x	x	x	x
PMH35	127 / 133	110 / 114	x	x	x	x	x	x	x	x	x	x	x	x	160 / 170	x	x	x
PMH36	125 / 127	110 / 140	x	x	x	x	x	x	x	114 / 114	x	x	x	x	172 / 176	x	x	x
PMH37	127 / 131	112 / 138	x	143 / 143	x	x	x	x	x	x	x	x	136 / 136	x	170 / 172	x	x	x
PMH38	139 / 149	150 / 150	x	x	x	x	x	x	160 / 160	114 / 114	x	x	x	x	160 / 200	x	x	x
PMH39	149 / 149	140 / 140	x	x	x	x	x	x	x	x	x	x	x	x	160 / 200	x	x	x
Total no. A.	10	11	2	4	4	3	5	4	6	7	6	4	4	5	8	4	3	3

4. Discussion

4.1 DNA extraction and evaluation

My study showed that DNA isolated from fresh tissue samples yielded good quality of DNA. However, hair samples yielded very low amplifiable DNA quality and dry skin samples did not yield amplifiable DNA quality. In the case of hair samples, this may be because of lacking hair follicles in the hairs. According to Alpers *et al.* (2003) and Piggott (2005) DNA quality of samples may be influenced by moisture, temperature, UV radiation, preservation method and sample age. Although it was unknown the upper limit of the viability of hair samples preserved in ethanol, my study showed that hair samples preserved in 96 % ethanol yielded poor quality DNA after 1 year storage. However, in my pilot study, DNA extracted from the red fox hair samples stored in paper envelopes at room temperature yielded good quality of amplifiable DNA after 2 years of collection (data not shown). Piggott (2005) found a significant reduction in DNA quality extracted from the red fox scats and brush-tailed rock- wallaby scats after 3 months of sampling preserved in paper envelopes. Similarly, Constable *et al.* (2001) found that Gombe chimpanzees hair samples frozen in liquid nitrogen and scat preserved in 100 % ethanol did not yield useful DNA after 1 year storage. DNA extracted from Chelex100 method was found very unstable and degraded within a week when stored at 4°C. However, I stored hair DNA stock at -80°C and it was repeated thawing and freezing (at -80°C) process seven times within a month to check DNA storage viability. Simultaneously, same DNA stock was tested on multiplex PCR that observed successful amplification till one month. According to researcher's experienced, addition of DNA preservative reagents may helpful to prevent DNA from degradation and increased storage viability of DNA (Kim Præbel, personal communication). Osmundson *et al.* (2013) focused several important considerations to obtain good quality of DNA and long term storage such as selection of DNA extraction methods, sample type and downstream applications. In my study, I used the NaOH DNA extraction methods for the extraction of DNA from dry skin samples but this method did not yield amplifiable DNA quality. The lower DNA yield suggests that the extraction method may not be suitable itself or needs the better DNA cleanup process.

4.2 PCR amplification

PCR assays developed in my study were highly reliable due to positive result obtained from fresh tissue samples of pine marten (Figure 3). However, most of the hair samples and dry skin samples did not amplify PCR multiplex markers. In the case of hair DNA extraction, I tested several extraction methods (describe in method section) beginning with a Chelex 100 developed by Suenaga and Nakamura (2005), that provides somehow better quality DNA. This extraction method enabled me to extract hair DNA that was suitable for PCR amplification and also gave me an opportunity to develop a successful PCR reaction protocol. My study showed higher PCR amplification when freshly extracted DNA was used. However, the lower successful PCR amplification obtained when stored DNA was used for PCR due to degradation of DNA and presence of reagents that inhibit PCR amplification. The entire DNA extracted from hair samples were degraded, therefore failed to PCR amplification. Frosch, Haase and Nowak (2011) showed lower amplification success in Eurasian beaver hair sample than tissues. In contrast, Constable *et al.* (2001) showed higher amplification success in Gombe chimpanzees hair samples than scats. On the other hand, DNA extract from dry skin did not amplify PCR program due to poor DNA quality. I performed phenol: chloroform: isoamyl alcohol and Wizard Genomic DNA cleanup kit to increase initial DNA quality for the dry skin samples. However, DNA isolated from dry skin was not successfully amplified by PCR. Constable *et al.* (2001) and Osmundson *et al.* (2013) used GeneClean kit to increase initial DNA quality of scats and PCR inhibitor kit to purify fungal and oomycate DNA respectively. In contrast, DNA extracted from Reindeer skin (similar type of samples like dry skin) using NaOH method (Wang, Qi & Cutler 1993) reported successful PCR amplification and genotyping success (R. Wilson, personal communication). My study showed that DNA containing a lot of PCR inhibitors and degraded DNA could not successfully amplified by PCR.

4.3 Microsatellite panels (markers)

The main objective of this study was to develop a multiplex panel from the microsatellite markers used for mustelid species for the study of population genetic structure in Norwegian pine marten. Here, 21 microsatellite markers including one sex identifying marker were selected and tested on tissue and hair samples in singleplex reaction. Among the 21 markers, 17 microsatellite markers and one sex identifying marker successfully amplified in PCR multiplexes panels. However, various problems associated with multiplex PCR such as lack

of amplification of loci and difficulties in reproducing result occurred during the optimization process. For a successful PCR multiplex assay, Henegariu *et al.* (1997) and Markoulatos, Siafakas and Moncany (2002) suggested to focus on some important conditions such as concentration of the PCR buffer, cycling temperature, the concentration of primers and proportion of $MgCl_2$ and deoxynucleotide (dNTP). In my designed PCR multiplex panels, I increased the concentration of relative primers, $MgCl_2$ and bovine serum albumin (BSA) in PCR reaction volume and obtained better intensity and good clarity of the peaks. Similar to Markoulatos, Siafakas and Moncany (2002), I found that concentration of Mg^{2+} increased PCR yield and BSA increased the efficiency of PCR. The amount of DNA extracted from all types of samples was low, thus I lowered annealing temperature at 54°C from 64°C and obtained efficient and specific amplification of multiplex markers after several trials. The successful amplification of multiplex panels in my study was observed in fresh tissues sample PMT1. The amplified product yielded DNA fragment between 100 bp to 350 bp compared with the 100 bp DNA ladder (Figure 3). However, only a few primers were amplified on hair samples and no amplification of primers occurred on museum specimen samples. This could be due to low concentration and poor quality of DNA as well as the presence of PCR inhibitors in the DNA extract. It was observed that the sex identifying marker K9Amelo successfully amplified in tissue sample PMT1.The K9Amelo marker amplified female homozygous allele with consistent allele size 180 bp and male heterozygous alleles with consistent alleles sizes 180 and 184 bp. However, one of the tissue sample PMT4 observed amplification of male heterozygous alleles with alleles sizes 180 bp and 190 bp (Table 5). On the other hand, Manivannan (2013; unpublished) found female homozygous allele sized 204 bp and male heterozygous alleles sized 190 and 204 bp in Norwegian red fox.

Genotyping success rate

In my study microsatellite markers were successfully grouped into 3 different multiplex panels to achieve genotyping success and identification of alleles. The genotyping success rate of fresh tissues was significantly higher (79%) in compared to hair samples (32%). Similar studies carried out on pine marten and other carnivores have reveled that genotyping success rate using hair follicles varied between 45.5 % and 76.6 % (Balestrieri *et al.* 2011; Davoli *et al.* 2013; Ruiz-González *et al.* 2013; Sheehy *et al.* 2014; O'Mahony, Turner & O'Reilly 2015). The genotyping success depends not only on successful amplification of

markers in PCR reaction but also on the quality of the DNA (Manivannan 2013; unpublished). A large number of hair samples in my current study did not provide individual information on genotyping due to degradation of the DNA, therefore required a large number of hair samples to develop a method for pine marten microsatellite panels and to conduct the several trials to come up in the conclusion. However, one of the hair samples showed very high percentage (88.7 %) amplification of markers on genotyping (Table 5, PMH9). This could be due to contamination with the positive tissue sample during genotyping. Sloane *et al.* (2000) and Frosch, Haase and Nowak (2011) used single hair follicles and even different laboratory for the DNA extraction to avoid potential contamination. Similar to Selkoe and Toonen (2006), genotyping result of my study showed a range of allele 2 to11 per locus (Table 5).

In my study, I used 1 µL DNA template isolated from all types of samples in 15 µL PCR reactions. However, Sloane *et al.* (2000), Frosch, Haase and Nowak (2011), Osmundson *et al.* (2013) and O'Mahony, Turner and O'Reilly (2015) used 2.5 to 8 µL DNA extracted using Chelex protocol in 10-15 µL PCR reaction. The evaporation of initial DNA extraction, some modification of the PCR protocol and additional PCR cycles may be improved genotyping success (Kim Præbel, personal communication). Pertoldi *et al.* (2014) purified PCR products with NucleoSpin Extract II that produced high genotyping success on different types of pine marten samples. However, purification of PCR products did not conduct in my study. Selkoe and Toonen (2006) showed that hair, skin and scat samples have a higher genotyping error than that of properly preserved tissue samples. All these results indicated that non-invasive samples like skin, hair and scat often required an intensive protocol for successful genotyping.

5. Conclusion

In my study, I successfully developed a microsatellite panel useful for further population genetic study of pine marten in Norway using 17 microsatellite loci. DNA was successfully extracted from tissue samples. However, hair samples yielded a low amount of DNA due to lack of follicles in the hairs. Therefore, I would recommend sufficient amount of hair samples directly pooled from the individual pine marten to test the reliability of designed primers for the genetic study of Norwegian pine marten based on hair samples in future. In addition, I would also recommend performing important steps such as examination of hair follicles in the hairs and species identification for the originality of samples for further analysis and microsatellite genotyping. The extraction of the DNA from the hair samples may be maximized by optimizing collection method, storage and extraction methods. Therefore, a well-designed and clear study plan should be made before starting field and laboratory work. Such a design was lacking in my study. The PCR multiplex design in this study provides successful genotyping on tissues samples and in a few hair samples. Hence, the PCR reaction and microsatellite multiplex panels can be used in future studies of pine marten. In this study, I also found that the samples stored in paper envelopes at room temperature yielded better DNA quality than the samples preserved in ethanol. Of the methods used, the Chelex method is recommended for extraction of DNA from hair samples while Isolate-II Genomic kit is recommended for tissue samples. Future research should focus on long-term storage viability of DNA and effective techniques to increase DNA concentration in the stock extracted from hair samples. Particularly, my study addressed the need for further knowledge on multiplex primers design, development of PCR reaction program and also evaluation of DNA extraction methods. The optimization of microsatellite panels might be possible to test universal fluorescent tagged PCR primers to minimize the cost associated with microsatellite genotyping on hair samples.

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