A forensic DNA profiling system for Northern European brown bears (*Ursus arctos*)

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ABSTRACT

A set of 13 dinucleotide STR loci (G1A, G10B, G1D, G10L, MU05, MU09, MU10, MU15, MU23, MU26, MU50, MU51, MU59) were selected as candidate markers for a DNA forensic profiling system for Northern European brown bear (*Ursus arctos*). We present results from validation of the markers with respect to their sensitivity, species specificity and performance (precision, heterozygote balance and stutter ratios). All STRs were amplified with 0.6 ng template input, and there were no false base genotypes in the cross-species amplification tests. The validation experiments showed that stutter ratios and heterozygote balance was more pronounced than in the tetranucleotide loci used in human forensics. The elevated ratios of stutter and heterozygote balance at the loci validated indicate that these dinucleotide STRs are not well suited for interpretation of individual genotypes in mixtures. Based on the results from the experimental validations we discuss the challenges related to genotyping dinucleotide STRs in single source samples. Sequence studies of common alleles showed that, in general, the size variation of alleles corresponded with the variation in number of repeats. The samples characterized by sequence analysis may serve as standard DNA samples for inter laboratory calibration. A total of 479 individuals from eight Northern European brown bear populations were analyzed in the 13 candidate STRs. Locus MU26 was excluded as a putative forensic marker after revealing large deviations from expected heterozygosity likely to be caused by null-alleles at this locus. The remaining STRs did not reveal significant deviations from Hardy–Weinberg equilibrium expectations except for loci G10B and MU10 that showed significant deviations in one population each, respectively. There were 9 pairwise locus comparisons that showed significant deviation from linkage equilibrium in one or two out of the eight populations. Substantial genetic differentiation was detected in some of the pairwise population comparisons and the average estimate of population substructure (FST) was 0.09. The average estimate of inbreeding (FIS) was 0.005. Accounting for population substructure and inbreeding the total average probability of identity in each of the eight populations was lower than 1.1 × 10−2 and the total average probability of sibling identity was lower than 1.3 × 10−4. The magnitude of these measurements indicates that if applying these twelve STRs in a DNA profiling system this would provide individual specific evidence.

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1. Introduction

DNA markers such as microsatellites have been extensively used in conservation genetics to study population diversity, impact of genetic drift and level of inbreeding in a variety of species [1].

One of the best studied mammalian species in conservation genetics is the European brown bear (*Ursus arctos*) [2]. Most data used in recent wildlife genetic studies of brown bear are from genotyping a collection of dinucleotide STRs that were isolated from brown bear and black bear (*Ursus americanus*) [3,4]. The development of non-invasive genetic sampling techniques has allowed sampling of living populations of large carnivores like the brown bear [3,5]. Non-invasive genetic sampling techniques open for long-term genetic monitoring of threatened carnivores that
occur at low densities. Capture-mark-recapture (CMR) analysis allows important parameters such as abundance, survival and migration to be studied [6,7]. As part of the population management of Northern European brown bear in Norway their abundance have been monitored since 2006 by use of CMR analyses of data from non-invasive samples typed in a set of dinucleotide STRs (see e.g. [8]).

The brown bears of Northern Europe are listed as threatened, but are often involved in conflicts with humans, livestock depredations and illegal hunting. When investigating wildlife crime, genetic analyses of sample materials could provide species specific identification of bear. Furthermore, if using a set of bear specific STRs, forensic genetic analyses would have the potential to provide individual specific bear profiles from a variety of sample materials and provide a means for traceability of bear products (e.g. food, trophy objects and medicine). Experience from population management of brown bears in Norway indicates that approximately 65–70% of non-invasive sample materials may be successfully typed in more than 6 STR loci [8]. Similar success rates when analyzing non-invasive sample materials have been reported by others [9].

Genetic identification of individuals by use of a DNA profiling system would rely on relevant reference data. The lack of such reference data may be a limitation when developing DNA profiling systems for large carnivores. However, these existing dinucleotide markers commonly used in wildlife population management may be used in a forensic DNA profiling system if the necessary allele frequencies from living populations could be retrieved in cooperation with population management laboratories. Dinucleotide STRs are widely used in population monitoring and conservation genetic studies of brown bear [1]. Tetranucleotide STRs are expected to have less stutter and less difference in heterozygote balance than dinucleotide STRs, and they are thus, the preferred markers in human forensics. However, one reason why dinucleotide STRs are commonly used in conservation genetics is that these markers has proven to work well in sample materials like faeces and hair due to relatively short amplicon sizes [1,3,8–10].

European laboratories that monitor bear populations already use very similar sets of dinucleotide STRs [10–12] while tetranucleotide STRs, on the other hand, are not used at all for DNA analysis of brown bear. Thus, a selection of dinucleotide STRs seems to be the preferred forensic markers to include in a European bear DNA profiling system. The ISFG recommendations, although pointing out the benefits of using tetranucleotide STRs, support the use of dinucleotide STRs if they are already in wide use in a non-human species [15]. Several recommendations regarding validation and use of non-human DNA in forensic genetics have been suggested to justify their application as evidence in court [13–15]. Such validation studies, that demonstrate the performance of any new markers, may be particularly important if applying dinucleotide STRs instead of the tetranucleotide STRs [15].

One aim of this study were to perform the recommended validation tests on thirteen dinucleotide microsatellite markers (G1A, G10B, G1D, G10L, MU05, MU09, MU10, MU15, MU23, MU26, MU50, MU51, MU59) commonly used for bear population management and conservation genetics. The validation tests could aid in the selection of markers for a forensic DNA profiling system for the brown bear in Northern Europe. The validation tests included species specificity testing, measurements of sensitivity as well as measurements of precision, stutter and heterozygote balance. Selected common alleles from all STR loci were sequenced to explore the allelic size variation at the sequence level. Another aim of this study was to provide allele frequency distributions as well as relevant forensic genetic parameters for the selected markers from eight bear populations in Northern Europe.

2. Materials and methods

2.1. Population material from eight brown bear populations in Northern Europe

The individual profiles in the population material are from samples collected in specific areas in Northern Europe (n = 479). Fig. 1 shows a map of Northern Europe with the approximate location of each of the eight populations indicated (P1–P8). A total of 290 of the individuals were from Norway, of which 233 individual profiles were obtained by typing non-invasive samples (fecal scats and hair) collected in the field as part of the monitoring of bears in Norway from 2006–2009, and 57 were obtained from legally shot bears in the same period (tissue and/or blood). The individuals were from four geographically separated areas; North-eastern Norway (P1, n = 74), North-western Norway (P2, n = 34), Middle Norway (P3, n = 81) and South-eastern Norway (P4, n = 101). The individuals from Middle Sweden were collected in 2009 in the county of Västerbotten (P5, n = 84) and the individuals from Finland were from the Kainuu area (P7, n = 44) and collected during 2005–2008. The sample materials from Sweden and Finland were scats collected in the field. The individuals from Russia were from two areas; the Pinega Strict Nature Reserve in Archangelsk (P6, n = 27) and Karelia (P8, n = 35). The sample materials were scats collected in the field in Pinega during 2005–2008 and tissue samples from legally shot bears in Karelia in the period 2005–2007.

2.2. DNA extraction, PCR amplification and STR analysis

DNA was extracted from hair-roots and tissue using the Qiagen DNeasy Tissue kit (Qiagen) and from faeces using the Invitrogen Stool kit (Invitrogen). Faeces were stored in stool collection tubes with DNA stabiliser (Invitrogen) or in plastic bags and kept at −20 °C until DNA extraction. The hair samples were stored dry and dark in paper envelopes until DNA extraction. No quantification of DNA concentrations was performed on the extracted samples. Instead,

![Fig. 1. The figure shows a map of northern Europe (including Norway, Sweden, Finland and Russia). The borders are shown as black lines, forest areas as green, scrub/bush and cropland as light green, tundra and mountains as brown and water as blue. The sample location of each of the eight bear populations, P1–P8 (with number of individuals in brackets) are indicated on the map. P1 (74); North eastern Norway n = 74, P2; North-western Norway n = 34, P3; Middle Norway n = 81, P4; South-eastern Norway n = 101, P5; Middle Sweden n = 84, P6; Pinega in Russia n = 27; P7; Kainuu in Finland n = 44, P8; Karelia in Russia n = 35. Estimates of the total number of bears in each of the four countries are given in blue below the name of the country. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](attachment:image)
the results from the two first STRs analyzed (MU05/MU23 or MU09/MU10) were used to roughly judge the amount of useful template DNA in each sample. Based on these first results the samples that were negative or judged to be of too poor quality for further analysis were removed. In the samples with either very strong or weak results the amount of template DNA used were modified to optimize the PCR amplification of the following STRs. All individuals in the eight populations were analysed in the 13 STRs: G1A, G1B, G1D, G1L, MU05, MU09, MU10, MU15, MU23, MU26, MU50, MU51 and MU59. These STRs had been previously cloned and characterized by others [3,4]. The modified forward and reverse primer sequences used in this study and the reference numbers to each of the corresponding loci from Genbank are given in Table 1. A short 5' tail [16] has been added to the reverse primer in seven of the thirteen primer pairs (see Table 1). PCRs were performed in 10 μl reaction volumes containing 1× PCR Gold buffer (ABI), 200 μM dNTP (Eurogentec), 1.5 mM MgCl₂ (ABI), 0.5 μM of each primer (MedProbe Inc.), 1 U AmpliTaqGold DNA polymerase (ABI), 1× BSA (NEB) and 1 μl template DNA. PCR conditions for loci G1A, MU10, MU05, MU09, MU23, MU50, MU51, MU59 and G1L were 10 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, and final extension for 15 min at 72 °C on an ABI 2720. PCR conditions for loci G1D, G10B, MU15 and MU26 were 10 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, and final extension for 5 min at 72 °C. Capillary electrophoresis was carried out on an ABI 3730 and the PCR fragments were analyzed in GeneMapper 4.0 (ABI). Allele sizes were measured using Genescan 500LIZ standard.

Duplicates of samples from four different individuals that together represented the common alleles at each marker were included as positive controls in all runs. Negative controls were included for every 7th sample. The positive controls were used to adjust for between-run-variation, and manual inspection of chromatograms was included as part of the final allele designations. All samples were typed at least two times by independent PCR runs followed by analysis on ABI 3730. Any sample typed as a homozygote genotype at any locus was confirmed by a minimum of three replicates (peak height threshold values >300RFU). The laboratory routinely types bear STRs for population management purposes. The extraction of samples and the analysis of the STRs are accredited according to the EN ISO/IEC 17025 standard (Norwegian Accreditation: Test 139).

A test of a multiplex PCR combining eight of the validated loci into one single PCR was carried out using Qiagen multiplex PCR-kit as described by the manufacturer's protocol except using 2 μl template and a total reaction volume of 10 μl. The concentration of each primer was 0.2 μM, except MU10 (0.4 μM) and MU59 (0.8 μM), and the PCR conditions were as in the singleplex PCRs. Loci MU09, MU10, MU23 and MU59 were labeled with FAM, MU05 with NED, MU50 with PET, MU51 and G1L with VIC (see Table 1). The multiplex was tested in sample extracts from tissue (1–2 ng template), hair (2–4 ng template) and faeces (unknown concentration of bear DNA) that previously had been successfully genotyped using singleplex PCR and shown to be single source samples originating from different individuals.

2.3. Sensitivity, species specificity, precision, stutter ratios and heterozygote balance

Two DNA samples (positive control samples) were used to test the effect of different template concentrations when amplifying the thirteen STR loci in singleplex PCRs. The following template input was tested in 10 μl reaction volumes: 30 ng, 20 ng, 15 ng, 10 ng, 5 ng, 2 ng, 0.6 ng, 0.4 ng, 0.3 ng, 0.2 ng, 0.03 ng and 0.02 ng.

All 13 markers were tested for cross-species amplification against two DNA samples from each of nine wild and domesticated...
animals as well as humans. The following species were included: elk (Alces alces), reindeer (Rangifer tarandus), wolverine (Gulo gulo), eurasian lynx (Lynx lynx), wolf (Canis lupus), hare (Lepus timidus), red deer (Cervus elaphus atlanticus), domesticated cat (Felis catus), red fox (Vulpes vulpes), and human (one male and one female). Another bear species, the polar bear (Ursus maritimus), was also included in the species tests. Two brown bear DNA samples (DNA extracted from tissue) was used as positive controls, PCR and STR analysis was performed as described in Section 2.2.

Measurements of between-run precision were obtained from all candidate STRs by ≥30 independent amplifications and subsequent runs of two heterozygote positive controls. Measurements of stutter ratio and heterozygote balance were also obtained from these runs. Within-run precision was measured in a sample from an individual with a heterozygous genotype at locus G10L and a homozygous genotype at locus G10B. Stutter ratio was calculated by dividing the peak height (RFU) of the stutter peak in position-1R (one repeat less than the true allele) by the peak height of the true allele. Paired students t-test was used to test for significant differences in stutter ratios between alleles within a locus. Heterozygote balance was calculated by dividing the peak height (RFU) of the short allele by the peak height of the longer allele. Heterozygote balance was calculated in this manner to give information about the direction of the imbalance.

2.4. DNA sequencing of tandem repeat arrays and immediate flanking sequences

The tandem repeat array and the immediate upstream and downstream sequences at each of the thirteen loci was analysed by DNA sequencing. PCR products amplified from individuals that were homozygous for common alleles were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) as recommended by the manufacturer. Forward and reverse PCR-primer were used as sequencing primers in forward and reverse sequencing reactions, respectively. Forward and reverse sequences from each sample were aligned by use of Sequencher 4.7 software. The allele sequences from a locus were aligned and, finally, the sequence and size variation within each locus was determined by manual inspection.

2.5. Additional PCR tests of locus MU26

New pairs of primers were applied to amplify an extended region upstream and downstream of the repeat array at locus MU26. The primers used were as follows; two different forward primers, each tested in combination with the same reverse primer (F1a = 5’ CGGCTTGGAGATAGGACAGA, F1b = 5’ TAAAGAGGGACAGTGGAT and R1 = 5’ GCCCTTTTACATTAGGTGAT) as well as a second forward and reverse primer set (F2 = GCCTTAATGACAGTTC, R2 = TCAATTAAATGAGCAGA). Samples were amplified using reaction conditions as described in Section 2.2. Four samples that had previously been successfully amplified at locus MU26 using the standard primers were used as positive controls. Samples from ten individuals that were previously successfully amplified in all loci but MU26 (putative null-allele homozygote individuals) were amplified in three different PCR tests using primers F1a and R1, F1b and R1, F2 and R2. PCR products were visualized on agarose gels and manually typed as positive (a clear PCR band of expected size) or negative (no PCR product).

2.6. Analysis of data

The GDA software v1.0 [17] was used for evaluation of Hardy–Weinberg equilibrium (HWE), linkage disequilibrium and population structure (Fis, overall and pairwise Fst). The estimation of population pairwise Fst values was also performed with Arlequin version 3.5.1.2 [18]. Bonferroni corrected significance levels were applied when testing HWE and linkage disequilibrium. Thus, when testing HWE the significance level was 0.0005 while when testing linkage disequilibrium the significance level was 0.00009. PowerStats (the gene count method) was used to obtain the observed allele frequencies and for the calculation of the forensic efficiency parameters [19]. API-Calc was used to estimate average probability of identity [20]. Micro-checker was used to test for presence of null-alleles and to discriminate between errors in allele frequency estimates caused by null-alleles, allele drop-out or stutter in a locus (MU26) with homozygous excess [21].

3. Results

3.1. Species specificity and sensitivity of the STR markers

The specificity tests of the 13 loci showed that PCR tests using non-bear templates resulted in no PCR-products at loci G1D, MU15 and MU51 while at other loci there were weak amplicons observed in some of the loci and species combinations. These weak amplicons had fragment sizes that were outside of the allele size range of the markers and could not be mistaken as bear genotype results. Template DNA from polar bear was successfully amplified with allele size ranges that partially overlapped those in brown bear profiles.

The sensitivity tests using different template input concentrations showed that all markers were successfully amplified when template input was in the range 30–0.6 ng. When decreasing the template input to 0.2 ng seven markers were successfully amplified while six of the loci showed drop-out of alleles (MU05, MU50, MU15, G10B, G1D, and MU09). Drop out was observed in all markers with template input less than 0.2 ng. All loci were successfully amplified and analyzed using template DNA extracted from tissue (frozen or dried), hair, blood and faeces.

3.2. Measurements of precision, stutter and heterozygote balance

The results from measurements of precision, stutter ratio and heterozygote balance are summarized in Table 2. The between-run measurements of precision revealed a standard deviation (S.D.) that were 0.16 bp or less in seven markers and from 0.16 to 0.20 bp in five markers while locus G10L revealed a standard deviation of 0.30 bp. There was no obvious difference in precision between alleles with different size within a locus. The within-run measurements of precision at loci G10L and G10B showed standard deviations of 0.16 bp and 0.08 bp, respectively. Fig. 2 a shows a chromatogram with results from locus MU23 and demonstrates separation of alleles differing by 1 bp (see also Section 3.3).

Stutter was observed in all markers as peaks at positions that were from one to several repeats less than the true allele (-1R, -2R, -3R etc.) and in some cases one repeat larger than the true allele (+1R). Fig. 2b and c shows the chromatograms from two individuals that are heterozygous and homozygous at loci MU59 and MU50, respectively. The figures illustrate the characteristic patterns of stutter observed in the dinucleotide markers tested. The stutters revealed decreasing peak heights with increasing size difference to the true allele in all markers. The stutter ratios (peak height of stutter/peak height of true allele) for peaks in position–1R (largest stutter peak) were recorded for short allele and large allele in all loci except MU05 and MU10. At these loci the stutter ratios were recorded for the short allele only since true alleles were separated by one repeat only in the control sample used for performance measurements. The median stutter ratios and upper 95% percentiles are given in Table 2. There was a significant difference in stutter ratio between the alleles within a locus with
smaller stutter ratios for the shorter allele. The median stutter ratios at the different loci ranged from 0.14 to 0.58 for the short allele and 0.37 to 0.75 for the larger alleles. The single largest stutter observed in any marker was 0.91 (MU59). A very small stutter peak was observed in position +1R in some cases and the average stutter ratio of these peaks were 0.09 (data not shown).

The variation in heterozygote balance was recorded in all loci. Heterozygote balance was calculated by dividing the peak height (RFU) of the short allele by the peak height of the larger allele to provide values that gives information about the direction of the imbalance. A heterozygote balance ratio less than one was rare. Thus, the shorter alleles were usually the ones with the largest peak heights, but ratios less than one were recorded in a few cases at loci G10L, MU09, MU23, MU50 and G1D (data not shown). The median values for heterozygote balance ratios for the thirteen STRs ranged from 1.13 at G1D to 2.18 at G10B. The median value from each locus as well as upper and lower 95 percentile is given in Table 2. The upper 95 percentiles of stutter and the lower 95 percentiles of heterozygote balance ratios did not overlap at any locus but MU09. This locus performed slightly less with no overlap between upper and lower 95.5 percentiles of stutter and heterozygote balance, respectively. We did not observe any heterozygote balance ratio larger than 3.3 (i.e. large allele approximately 30% of the peak height of the short allele).

### 3.3. Repeat numbers and repeat array structure in common alleles

Fifty alleles representing the most common alleles from all loci were sequenced. The alleles were aligned with the sequence data from Genbank and the repeats designated in accordance with the original characterization of the dinucleotide tandem repeat arrays at each locus [3,4]. The results from the sequence analysis with allele size, repeat numbers and repeat structure of the alleles from each of the loci are summarized in Supplementary File 1. Eight of the loci showed a simple tandem repeat array structure while loci MU50, MU10, MU23 and MU26 revealed compound repeat structures with (CT)\textsubscript{n}(GT)\textsubscript{m}, (TG)\textsubscript{n}(TA)\textsubscript{m}, (TG)\textsubscript{n}(AT)\textsubscript{m}(GT)\textsubscript{n}, and (TG)\textsubscript{n}(TA)\textsubscript{m}(TG)\textsubscript{m}, respectively. At locus G10L there was an insertion of TTGTCT within the repeat array that was not present in the Genbank sequence from black bear (Ursus americanus).

For ten loci we measured size differences that corresponded to the variation in repeats. However, for three loci (MU23, MU59 and G1A) additional differences affecting the allele sizes were revealed outside of the tandem repeat array. The sequence analysis of MU23

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**Table 2** Measurements of precision, heterozygote balance and stutter ratio.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Alleles/genotype\textsuperscript{a}</th>
<th>Mean (bp)\textsuperscript{b}</th>
<th>S.D. (bp)\textsuperscript{c}</th>
<th>Het. balance\textsuperscript{d}</th>
<th>Stutter ratio\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MU05 allele A</td>
<td>125</td>
<td>125.39</td>
<td>0.18</td>
<td>1.77 (1.39–2.72)</td>
<td>0.56 (0.62)</td>
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<td>MU05 allele B</td>
<td>127</td>
<td>127.51</td>
<td>0.17</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MU09 allele A</td>
<td>110</td>
<td>109.74</td>
<td>0.20</td>
<td>1.48 (0.76–2.64)</td>
<td>0.49 (0.63)</td>
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<td>MU09 allele B</td>
<td>116</td>
<td>116.01</td>
<td>0.20</td>
<td>–</td>
<td>0.60 (0.80)</td>
</tr>
<tr>
<td>MU10 allele A</td>
<td>149</td>
<td>149.92</td>
<td>0.12</td>
<td>1.73 (1.28–2.33)</td>
<td>0.53 (0.61)</td>
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<tr>
<td>MU10 allele B</td>
<td>151</td>
<td>152.11</td>
<td>0.10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MU23 allele A</td>
<td>166</td>
<td>166.32</td>
<td>0.18</td>
<td>1.36 (0.98–2.06)</td>
<td>0.54 (0.64)</td>
</tr>
<tr>
<td>MU23 allele B</td>
<td>173</td>
<td>173.65</td>
<td>0.20</td>
<td>–</td>
<td>0.67 (0.69)</td>
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<tr>
<td>MU50 allele A</td>
<td>120</td>
<td>120.59</td>
<td>0.19</td>
<td>–</td>
<td>0.54 (0.62)</td>
</tr>
<tr>
<td>MU50 allele B</td>
<td>124</td>
<td>124.79</td>
<td>0.19</td>
<td>1.61 (0.99–2.35)</td>
<td>0.74 (0.90)</td>
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<tr>
<td>MU51 allele A</td>
<td>139</td>
<td>138.94</td>
<td>0.17</td>
<td>–</td>
<td>0.32 (0.39)</td>
</tr>
<tr>
<td>MU51 allele B</td>
<td>149</td>
<td>149.78</td>
<td>0.16</td>
<td>2.01 (1.22–3.15)</td>
<td>0.54 (0.61)</td>
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<tr>
<td>MU59 allele A</td>
<td>240</td>
<td>240.29</td>
<td>0.10</td>
<td>–</td>
<td>0.52 (0.64)</td>
</tr>
<tr>
<td>MU59 allele B</td>
<td>256</td>
<td>256.54</td>
<td>0.09</td>
<td>1.53 (1.03–2.59)</td>
<td>0.75 (0.87)</td>
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<td>G10L allele A</td>
<td>174</td>
<td>174.17</td>
<td>0.30</td>
<td>–</td>
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<td>182</td>
<td>182.50</td>
<td>0.30</td>
<td>1.36 (0.67–2.86)</td>
<td>0.50 (0.58)</td>
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<td>G10L allele A\textsuperscript{f}</td>
<td>174</td>
<td>173.9</td>
<td>0.15</td>
<td>–</td>
<td>0.33 (0.38)</td>
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<td>182.25</td>
<td>0.16</td>
<td>1.55 (0.89–2.17)</td>
<td>0.44 (0.49)</td>
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<td>G1D allele A</td>
<td>129</td>
<td>129.50</td>
<td>0.11</td>
<td>–</td>
<td>0.40 (0.45)</td>
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<td>G1D allele B</td>
<td>133</td>
<td>133.51</td>
<td>0.10</td>
<td>1.13 (0.96–1.79)</td>
<td>0.45 (0.47)</td>
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<td>G10B allele A</td>
<td>98</td>
<td>97.34</td>
<td>0.13</td>
<td>–</td>
<td>0.37 (0.40)</td>
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<td>G10B allele B</td>
<td>110</td>
<td>109.82</td>
<td>0.15</td>
<td>2.18 (1.77–2.54)</td>
<td>0.62 (0.67)</td>
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<td>G10B hom B\textsuperscript{g}</td>
<td>110</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>G10B hom B\textsuperscript{g}</td>
<td>110</td>
<td>109.69</td>
<td>0.08</td>
<td>–</td>
<td>0.62 (0.68)</td>
</tr>
<tr>
<td>G1A allele A</td>
<td>181</td>
<td>181.06</td>
<td>0.16</td>
<td>–</td>
<td>0.57 (0.65)</td>
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<tr>
<td>G1A allele B</td>
<td>189</td>
<td>189.38</td>
<td>0.13</td>
<td>1.47 (1.08–2.09)</td>
<td>0.70 (0.73)</td>
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<tr>
<td>MU15 allele A</td>
<td>110</td>
<td>109.86</td>
<td>0.15</td>
<td>–</td>
<td>0.58 (0.63)</td>
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<td>MU15 allele B</td>
<td>116</td>
<td>116.13</td>
<td>0.14</td>
<td>1.55 (1.29–2.02)</td>
<td>0.74 (0.79)</td>
</tr>
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<td>MU26 allele A</td>
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<td>82.98</td>
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<td>–</td>
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<tr>
<td>MU26 allele B</td>
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<td>96.09</td>
<td>0.09</td>
<td>1.79 (1.34–2.30)</td>
<td>0.37 (0.39)</td>
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\textsuperscript{a} Alleles as designated with a size based nomenclature.
\textsuperscript{b} Mean value of allele sizes when measured with popz on AB3730.
\textsuperscript{c} Standard deviations (S.D.) from between-run measurements of \textsuperscript{c} 30 runs of a control sample.
\textsuperscript{d} Median heterozygote balance ratio with upper and lower 95 percentile in parenthesis.
\textsuperscript{e} Median stutter ratios of alleles with upper 95 percentile in parenthesis.
\textsuperscript{f} Within-run measurements of precision (S.D. = 0.16 bp), heterozygote balance and stutter ratio.
\textsuperscript{g} Within-run measurements of a sample with a homozygous genotype (S.D. = 0.08 bp).
revealed an indel of a G downstream of the repeat array (position 211, Genebank Acc. No. Y09645) that produced alleles with identical number of repeats that differ by 1 bp. This finding was in agreement with the size measurements that showed microvariation among alleles at this locus (Fig. 2a). Sequence analysis at locus MU59 showed that there was a 4 bp indel located in position 121 (Genbank Acc. No Y09649) while at locus G1A there was a 4 bp indel located in position 164 (Genbank Acc. No U22095) downstream of the repeat array. Both these indels contribute allele size differences that cannot be distinguished from size variation caused by variation in repeat numbers.

The Genbank sequences from the four “G-named loci” (see Genbank Acc. No in Table 1) were isolated from black bear. Our novel genomic sequences in the present study were from brown bear. Comparing Genbank sequences from the black bear with our sequences from brown bears revealed additional sequence differences in the DNA flanking the tandem repeat arrays at some of the loci. The brown bear sequences at locus G1A showed an insertion of an A in position 185. In the brown bear sequences at locus G10B we revealed a substitution (A/T) with a T at position 79 upstream of the repeat array. At the G1D locus, we detected an insertion of a C at position 148 downstream of the repeat array in the brown bear sequence.

Putative SNPs were detected at two loci (G10B, G1D), and in both cases the less frequent variant was observed in two or more individuals. The one observed at locus G10B was a transversion (A/C) in position 93 while the one observed in locus G1D was a transition (A/G) in position 134.
Table 3
Population data from eight Northern European brown bear populations.

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No. Alleles

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<th>Av. PI</th>
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<tr>
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</table>

3.4. Population data

A total of 479 individuals from eight Northern European brown bear populations were typed in 13 STR loci (G1A, G10B, G1D, G10L, MU50, MU09, MU10, MU15, MU23, MU26, MU50, MU51, MU59). Allele frequencies from each of the populations are given in Supplementary File 2. The expected and observed heterozygosity frequencies for all loci in each of the eight populations are given in Table 3. Locus MU26 showed a significant deficiency of heterozygotes in three of the populations (P1, P4 and P7), and additional studies of MU26 (see Section 3.5) suggested that null-alleles were present at this locus. Consequently, the MU26 locus was excluded as a putative forensic marker and allele frequency estimates and other forensic parameters are not presented for this locus.

The heterozygosity frequencies ranged from 0.54 (MU15, P4) to 0.95 (MU59) among the remaining twelve loci, although with a single exception for locus MU15 that revealed a heterozygosity frequency of 0.38 in P2. The mean heterozygosity in the eight populations ranged from 0.69 (P5) to 0.83 (P6). Deviations (p < 0.05) from Hardy–Weinberg equilibrium (HWE) were observed in 19 out of 104 tests. However, after Bonferroni-correction there were significant deviations in five out of the 104 tests (p < 0.0005, marked in bold in Table 3). The five tests with HWE deviations were at loci G10B and MU10 in populations from Kainuu (P7) and North-eastern Norway (P1), respectively, as well as the above mentioned three populations with significant deficiency of heterozygotes at MU26.

Tests for deviation from linkage equilibrium by comparing locus pairs across populations showed that sixteen percent of the 12 loci showed linkage disequilibrium (LD) at a significance level of p < 0.05 (data not shown). None of these locus combinations were consistent in linkage disequilibrium in all populations. After Bonferroni-correction of significance levels (528 tests, p < 0.00009) there were 9 pairwise locus combinations that remained significant (data not shown). Six of these significant deviations from LD were in pairwise comparisons of locus G10B to another locus in the Kainuu population (P7). However, locus G10B showed also a significant deviation from HWE in this population (see Table 3), and when controlling the HWE effect on the LD test (“the preserving genotypes” option in GDA) none of these deviations remained significant. The observed heterozygosity at MU59 was high and at same time stutter ratios were elevated (see Table 2). A possible genotyping error (a homozygous genotype with a large stutter designated as heterozygous genotype) could result in such a high heterozygosity. Although our genotyping protocol should be robust, we compared the observed number of heterozygous genotypes consisting of alleles that differed by only one repeat to the expected number of such genotypes. The results showed only sparse deviation between the numbers of observed versus expected genotypes. We also re-checked all the genotype results from the two most common heterozygous genotypes with alleles that differed by one repeat (246/248 and 248/250), and in all cases the shorter sized alleles showed the largest peak heights in the two independent analyses. Altogether, these results indicate that the high heterozygosity at MU59 is not caused by genotyping errors.

The overall FST-value was 0.09 (bootstrap confidence interval 0.07–0.10). The population pairwise FST-s were significant for all comparisons (p < 0.0001) and ranged from 0.008 (P7–P8) to 0.16 (P2–P4) (Supplementary File 3). Estimates of the inbreeding factor (FIS) as well as confidence intervals for FST are given for each of the populations in footnotes in Supplementary File 2 (MU26 not included). The inbreeding factor ranged from −0.064 (P2) to 0.044 (P8). Significant levels of inbreeding (lower confidence interval above 0) were observed in two of the populations (P5 and P8). The overall value of FIS was 0.005 (not including locus MU26). The overall value of FST across populations at MU26 was 0.22. However, this large value is likely caused by the presence of null-alleles rather than inbreeding (see also Section 3.5).

The number of alleles and mean heterozygosity at each of the loci in the total population as well as the average probability of identity at each locus (Av. PI) is given in Table 3. The total average probabilities of identity for each population (Tot. Av. PI in Table 3) refers to the probability of observing two copies of any profile in that population when applying the twelve STRs [20]. When estimating the total average PI we used the population specific allele frequencies and FST – values and the average FST – value. The largest total average PI in any population was 1.1 × 10–4. The largest total average probability of sibling identity in any population was 1.3 × 10–4. Some additional common forensic efficiency parameters (power of discrimination, power of exclusion, matching probability and typicalaternity index) are given for each of the populations in Supplementary File 2.

3.5. Heterozygote deficiency at locus MU26

We used Micro-checker 1.0 [21] to further explore the significant heterozygote deficit in MU26 observed in the three populations P1, P4 and P7 by comparing the expected and observed frequency of homozygotes within different allele size classes. The comparison revealed a larger than expected frequencies of homozygotes in most allele size classes in these three populations (data not shown). Furthermore, samples from 17 out of the 219 individuals in P1, P4 and P7 did not produce results in MU26 while they could be genotyped in all other loci. One reasonable explanation for the lack of results in these samples could be that they are from individuals that are homoygous for the null-allele.

New primers located 5’ and 3’ of the original forward and reverse primers were applied to amplify locus MU26 by use of three different PCR tests. Template DNA from ten individuals that were previously successfully amplified in all loci but MU26 (putative null-allele homozygote individuals) were used in an attempt to amplify locus MU26 by use of these new primers. Applying the three new PCR tests locus MU26 was successfully amplified in four positive controls while it could not be amplified in any of the ten individuals.

3.6. Octa-plex PCR for eight common STRs tested on brown bear samples

Tests of a multiplex PCR amplification showed that eight of the validated STRs may be combined and successfully amplified at sensitivity levels as low as 1 ng in a single reaction using primers identical to the ones used in singleplex PCRs. The genotypes from individuals amplified by use of template extracts from tissue, hair and faeces were identical to the genotypes at the eight loci when amplified in singleplex PCRs (Fig. 3).

4. Discussion

4.1. Species specificity and sensitivity of the STR markers

The test species represented animals that bears predate on or animals from where hair and scats may falsely be collected as bear samples. Humans were included to assure that contamination by human DNA, e.g. in the laboratory, would not produce false results. The results demonstrate that presence of DNA from the test species would not lead to any false positive results.

Although even more species may be tested, the results indicate that all the markers have high species specificity. Assignment of individual identity would depend on results from many STRs, but if testing only for presence of bear (a species specific test), positive
results (genotype results) from a smaller number of loci would provide strong bear specific evidence.

Several of the markers have been shown to produce genotype results in other bear species [4,22]. The loci were successfully amplified in polar bear when amplified with the primers from this study, and the sequence comparisons to black bear from Genbank at four loci (see Section 3.3) revealed few sequence differences. Thus, although the primers have not been tested in many bear species, it is likely that they would be successfully amplified in other bear species and should not be regarded as brown bear specific.

Traditional DNA quantification methods (spectrophotometric estimation) would fail to measure the amount of bear specific DNA in sample materials like faeces which also contain DNA from food and microorganisms. Neither would the amount of DNA degradation and presence of PCR inhibitors be detected by use of such DNA quantification methods. Development of a species specific qPCR assay could provide better estimates of template concentrations, thus, be a good addition to a bear DNA profiling system, but in absence of any qPCR method we have simply used the results from the two first STR loci typed to roughly judge the quality of the DNA extract (DNA-concentration, degree of degradation) in each sample. The measurements of sensitivity showed that there were no large differences among the markers validated. They all worked well in a large range of template concentrations. The use of two initial STRs to roughly judge the quality of the DNA extract would be unwise if there were any large differences in sensitivity among the markers. However, the similar sensitivity range of the validated STRs indicates, that if failing in the two initial STRs due to a low sample DNA concentration, it is likely that this sample will also fail in many of the other markers, and, thus, supports the use of results from the initial STRs as an indicator of template success rate in the following STRs.

The measurements of sensitivity as well as the other performance measurements (see Section 4.2) are from results using a singleplex PCR amplification as this is the current procedure used when analyzing the validated loci at our laboratory. While the aim of this study was not to develop new multiplex assays, a multiplex PCR that combine several of our validated and also widely used bear STRs into single PCR reactions would be valuable to both population monitoring and forensic use of these markers (less time consuming and more cost effective). A recent study has reported the performance of a multiplex PCR that included eight of the validated STRs in this study [9]. Likewise, we have shown that an octa-plex PCR consisting of eight of the most commonly used markers in conservation genetics and population monitoring may be successfully amplified in DNA extracts from tissue, hair and faeces, and at approximately same sensitivity levels as in the singleplex PCRs. This demonstrates that subsets of the validated loci may be combined into multiplex assays. Preferably, such multiplex assays should not perform much less than the singleplex methods, and the measured performance levels from the respective validated singleplex assays in this study should serve as reference values when evaluating performance and loci-balance in multiplex assays of the validated STRs.

4.2. Precision, stutter and heterozygote balance

There is a correlation between the size of the standard deviation and the power to discriminate between alleles of different size. If the bin range is equal to three standard deviations and the bin ranges do not overlap, 99.7% of identical alleles are sized within the same bin [23]. The measurements of precision in this study indicate that alleles with 2 bp size differences can be discriminated with a confidence of more than 99.7% in all loci. Seven of the loci also show a between-run precision that allows a similar high discrimination of alleles with only 1 bp size differences (3 × S.D. <0.50 bp). Within-run measurements of the least performing
locus, G10L, revealed that the precision improved considerably when eliminating the between-run factors (from 0.30 bp to 0.16 bp). A similar improvement was demonstrated at locus G10B (from 0.15 bp to 0.08 bp).

Allelic ladders are usually applied to control the between-run factors from affecting precision. In our study we have applied four positive controls as allelic ladders and the bin windows were adjusted according to the size measurements of the alleles in these control samples. Given the demonstrated precision and the typing procedure applied in our study, it is likely that any common alleles with size differences of 1 bp would be detected. Such size differences were observed at locus MU23, only. Thus, micro-variation (1 bp size variation) was not common in our North European brown bear populations at the other loci tested. The micro-variation at MU23 contributes to the size diversity at this locus, and we believe that if applying a similar analyzing system as in this study that has the power to discriminate between 1 bp size differences MU23 could still be included in a brown bear DNA profiling system.

Stutter is a common artifact inherent in STR amplification. The height of stutter peaks depend on the type of repeat units (di, tri, tetra, penta) and is expected to be much more pronounced in dinucleotides than in tetranucleotides. The heterozygote balance at dinucleotide loci is also expected to differ substantially from the ones observed in tetranucleotide loci with larger differences in peak heights between alleles [24–27]. In agreement with these expectations the stutter ratios observed in the thirteen dinucleotide STRs were considerably larger than those reported in tetranucleotide loci and they seem to increase with number of repeats within each locus. The imbalance between alleles in a heterozygous genotype was also substantially larger in dinucleotide loci and there was a direction of the imbalance with shorter alleles being the ones with largest peak heights.

The presence of three or more alleles in any of the markers would be required to distinguish between a mixture of two (or more) individuals and a single source sample (one individual). Although not tested in particular, the heterozygosity level of the validated markers supports that in a mixture of two or more unrelated individuals there would usually be three alleles in at least one of the loci. As in other DNA profiling systems consisting of a combination of STRs, the minimal number of individuals in a mixture may be estimated on basis of maximum number of alleles observed in any of the markers. However, given the elevated ratios of stutter and heterozygote balance in the dinucleotide loci, we believe that interpretation of the individual genotypes in a mixture would be difficult if not impossible.

There are two common types of genotyping errors in single source samples associated with large stutter ratios and large variation in heterozygote balance. One such error would be failure of detecting a drop-out of a larger allele. This would lead to mistyping a heterozygous genotype as a homozygous genotype. Such errors could occur if the interval between the homozygote peak height threshold (threshold for accepting a true homozygous genotype) and the analytical threshold (detection limit, usually 50 RFU) does not reflect the heterozygote balance ratios. The larger alleles are usually above 60% peak heights of the shorter alleles in dinucleotide loci and the homozygote peak height threshold used are about 150–200 RFU [25]. The measurements of heterozygote balance ratio in the dinucleotide STRs showed that peak heights of the larger alleles were usually in the range 40–70% of the shorter allele, but never less than 30% (a heterozygote balance ratio of 3.3). The more pronounced differences in allelic imbalance indicate that larger homozygote peak height threshold values should be applied when genotyping the dinucleotide loci. In our study we used 300 RFU to assure that a too narrow interval between homozygote peak height and analytical peak height thresholds did not lead to this kind of genotyping errors.

The other common type of genotyping error would be failure to distinguish between a heterozygous genotype where the smaller allele has the lesser peak height and a homozygous genotype with a large stutter. Such errors could occur at a locus if the values of heterozygote balance ratios and stutter ratios overlap, e.g. if the heterozygote balance ratio are less than one (shorter allele has lesser peak height than larger alleles in a true heterozygous genotype) and the stutter ratios are high (close to one). However, heterozygote balance ratios were usually well above one (Table 2), and the upper 95% percentiles of stutter and the lower 95% percentiles of heterozygote ratios did not overlap at twelve of the loci while one locus performed slightly less (MU09). Thus, usually the random variation in stutter and heterozygote ratios does not result in overlapping values. Genotyping should, however, be carried out manually by expert analysts with detailed knowledge of the expected heterozygote balance and stutter ratios in the STRs used. Improved confidence in allele designation may also be achieved by independent analysis of duplicates from each sample. A procedure, as applied in our study, where all samples are typed in duplicate and all homozygote genotypes are typed in triplicate would further limit the chance that imprecision, random variation of stutter ratios or heterozygote balance in a single typing would lead to errors in allele designation.

MU09 showed a small overlap between stutter and heterozygote balance values. Thus at this locus there are particular challenges when genotyping heterozygous individuals with alleles that differ by one repeat. If using markers like MU09 the expert analyst should, in our opinion, pay particular attention to allele designation of such genotypes. The heterozygote balance ratios at MU09 were in some cases less than one, but most true heterozygous samples would show a value larger than one in at least one of the two individual analyses of the sample, thus, recognized as a true heterozygous genotype (or if in doubt even typed a third time). In rare occasions a true a heterozygous genotype would show heterozygote balance values less than one in two independent analyses. If so, it would be regarded as a potential homozygote and typed independently a third time. The chance that a true heterozygous genotype would show heterozygote balance ratios that could be interpreted as stutter by chance in three independent analyses would be very rare. However, if this should occur, that all three analyses resulted in values that are in the overlap between stutter and heterozygote balance, the sample should simply be scored as an ambiguous genotype and not included in the DNA-profile given for this sample. We believe that such “challenging” loci may be used if the protocols and standard operating procedures documents the individual performance of the loci used and provide threshold guidelines to assure that if values of stutter and heterozygote ratio are at levels that results in ambiguous genotypes they are not included in the DNA-profile.

4.3. Variation of repeat numbers and repeat array structure

Allele designations used in the population genetic studies of brown bear are usually based on allele size measurements. The designation to a certain allele size depends on the primers and electrophoretic systems (as well as size standards and polymere) used at the laboratory. Thus, identical alleles are usually designated with different sizes at different laboratories. If a laboratory applies an in-house allelic ladder, any change in nomenclature would not itself improve the confidence in allele designation. However, it makes inter laboratory co-operation and sharing of population frequency data less complicated. Thus, a common nomenclature and inter laboratory calibration based on sharing of standard DNA samples would be a benefit for the conservation genetic community as well as for the use of these markers for forensic purposes.
The size variation in the alleles from ten of the STR loci validated in our study seemed to depend on variation of number of repeats, only. A nomenclature where allele designation is based on number of repeats in a given allele could therefore, as suggested by the forensic community [13,15], be a unique and better way to designate these alleles. Three loci showed indels in addition to the variation in number of repeats, and more alleles (allelic ladders) from all loci should be sequenced before details of the system for allele nomenclature at single loci are suggested. To make such a change to a consensus nomenclature valuable it should be a collaborative task in which laboratories that routinely genotype European brown bear participate and agrees on implementing a common nomenclature. Thus, our samples with the sequenced alleles are potential standards that may be used in an inter laboratory calibration which may represent the first steps towards a change to a common nomenclature.

The species differences revealed by comparison to the Genbank sequence from black bear as well as the polymorphisms discovered at some loci must be taken into account if designing new primers to amplify these microsatellites in brown bear. The species differences, if confirmed as fixed differences by typing more individuals from both species, have the potential to be utilized for species specific amplification of the markers.

4.4. Heterozygote deficiency at locus MU26

Null-alleles as well as genotyping errors caused by stutter (interpreting a heterozygous genotype as a homozygous genotype with large stutter) or drop-out of large alleles could lead to higher than expected presence of false homozygous genotypes. However, when testing the expected versus observed distribution of homozygous genotypes in P1, P4 and P7 within allele size classes at locus MU26 the frequencies of homozygous genotypes were larger than expected in most of the allele sizes. If the homozygote excess was caused by scoring error due to stutter or large allele drop-out one would expect a deficiency and excess of particular genotypes [21]. Thus, there was no evidence that the heterozygote deficiency was caused by genotyping errors.

Heterozygote deficiency in all size classes could also result from inbreeding. However, inbreeding is expected to affect the whole genome, and there was a substantial difference in the inbreeding coefficient observed at MU26 (0.22) and the average inbreeding coefficient from the other 12 microsatellite markers (0.005). Thus, although there is a certain degree of inbreeding in some populations, we believe that null-alleles are the main cause of the extreme heterozygote deficit observed at MU26.

A common cause of null-alleles is polymorphisms located at the primer sites [28]. However, there was no amplification of locus MU26 when re-amplifying this locus by use of new primer pairs and template DNA from putative null-allele homozygous individuals. Thus, the cause of null-alleles remains unknown. A large deletion of the entire locus may, however, be a possible explanation.

4.5. Population data

The locus heterozygosity and number of alleles observed at the twelve loci in the total population (see Table 3) indicates that all loci are highly polymorphic. In wildlife species like the brown bear the ideal conditions that must be assumed for Hardy–Weinberg equilibrium (e.g. infinite population size, random mating) does not exist. Nevertheless, the tests for HWE demonstrated that there were few significant deviations from expected heterozygosity at twelve of the loci, while it lead to the identification of null–alleles at locus MU26. None of the remaining twelve loci were in linkage disequilibrium in all populations. Thus, in lack of any mapping information, the assumption that the loci were not closely linked was not invalidated. There were moderate levels of population substructure (average FST 0.09) and significant levels of inbreeding were revealed in only two populations. This suggests that a general theta adjustment of 0.09 could be used in match probability estimates in the Northern European brown bear meta-population. An inclusion of an estimate of inbreeding in the match probability could, as suggested in Dawney et al. [29], be included at homoyzgote loci. However, if the alternative hypothesis implies that the alternative matching individual is from the same geographical location we believe that a sibling match probability, representing the most conservative match estimate, could also be reported since home range overlap is positively correlated with relatedness in brown bears [2].

In general, the level of population structure increased with distance between the populations, although this was not consistent for all single comparisons. The population pairwise FST’s (Supplementary File 3) indicated close relationship between the populations of Kainuu and Russian Karelia (P7 and P8, FST = 0.0086). In contrast, there was substantially larger differentiation between North-eastern Norway and North-western Norway (P1 and P2, FST = 0.1033) that may not have been caused by distance alone. The estimates of population substructure and inbreeding were included when estimating the total average probability of identity and total average probability of sibling identity [20]. The magnitude of these estimates indicates that a DNA profiling system applying the twelve STRs (G1D, G10B, G10L, G1A, MU05, MU09, MU10, MU15, MU23, MU50, MU51 and MU59) would provide individual specific DNA profiles.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2012.03.002.

References


