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SHORT COMMUNICATION

Y-chromosomal testing of brown bears (*Ursus arctos*): Validation of a multiplex PCR-approach for nine STRs suitable for fecal and hair samples

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Abstract

High-resolution Y-chromosomal markers have been applied to humans and other primates to study population genetics, migration, social structures and reproduction. Y-linked markers allow the direct assessment of the genetic structure and gene flow of uniquely male inherited lineages and may also be useful for wildlife conservation and forensics, but have so far been available only for few wild species. Thus, we have developed two multiplex PCR reactions encompassing nine Y-STR markers identified from the brown bear (*Ursus arctos*) and tested them on hair, fecal and tissue samples. The multiplex PCR approach was optimized and analyzed for species specificity, sensitivity and stutter-peak ratios. The nine Y-STRs also showed specific STR-fragments for male black bears and male polar bears, while none of the nine markers produced any PCR products when using DNA from female bears or males from 12 other mammals. The multiplex PCR approach in two PCR reactions could be amplified with as low as 0.2 ng template input. Precision was high in DNA templates from hairs, fecal scats and tissues, with standard deviations less than 0.14 and median stutter ratios from 0.04 to 0.63. Among the eight di- and one tetra-nucleotide repeat markers, we detected simple repeat structures in seven of the nine markers with 9 to 25 repeat units. Allelic variation was found for eight of the nine Y-STRs, with 2 to 9 alleles for each marker and a total of 36 alleles among 453 male brown bears sampled mainly from Northern Europe. We conclude that the multiplex PCR approach with these nine Y-STRs would provide male bear Y-chromosomal specificity and evidence suited for samples from conservation and wildlife forensics.

Keywords: Y-chromosome, Wildlife forensics, Microsatellite, population genetics, conservation genetics, Non-invasive genetic sampling

1. Introduction

The brown bear (*Ursus arctos*) is an established model species in conservation genetics [1] and has been extensively studied using both maternally inherited mitochondrial DNA (mtDNA) and biparentally inherited autosomal STR-markers (e.g. [2-12]).Y-linked markers are important in population genetics since they provide information on the male inherited lineages. Y-STR testing may provide important information for a number of different applications including paternity testing, forensic evidence examination, conservation genetics, population and geographic origin assignment as well as studies of migration patterns. Bi-allelic loci like Y-SNPs and multi allelic loci like Y-STRs are two broad classes of DNA markers that have been used to examine Y-chromosome diversity. Results from combinations of the lower resolution Y-SNPs are usually classified into haplogroups while combination of alleles from the multi-allelic Y-STR loci are characterized as haplotypes [13]. In humans and primates Y-markers have been used to study e.g. population genetics, migration, social structures, and reproduction (e.g. [14, 15]) and Y-markers identified in domesticated animals have been applied in phylogenetic studies, e.g. in sheep, horses and canids (e.g. [16-20]). In human forensic science, Y-markers have been a helpful tool to investigate the geographical/ethnical ancestry of the DNA evidence [21, 22]. Despite the advantage of using a non-recombining marker in this type of forensic application, in wildlife forensic science the use of autosomal STR and mtDNA markers [6, 23-27] is still more common than the use of Y-markers [28] for determining the geographic origin of an unknown sample.

Recently, we identified multiple Y-STR loci from five Y-linked scaffolds in brown, polar and American black bear genomes. Nine of these Y-STRs were applied in a phylogenetic and phylogeographic study of brown, polar, and black bear [29]. Previously, we have developed a DNA profiling system based on autosomal STRs that are commonly used for bear conservation and in management as well as in forensic cases involving bears [6, 30]. We have also developed a sensitive and specific multiplex PCR assay for sex identification in non-invasive samples from bears [31]. Our aims for this study was to investigate the gene diversity in each of the nine Y-STRs in a large population mainly representing northern Europe. In addition our goal to establish a validated DNA profiling system for bear Y-chromosomal STRs suitable for typing sample materials used in brown bear conservation genetics and wildlife forensics. Thus, we have here performed tests for gender and species specificity, measurements of sensitivity and precision for all the nine bear Y-STRs in agreement with recommendations from ISFG [32]. Also, we investigate tandem repeat structure and allele size variation by DNA sequencing alleles from all loci. Finally, our validation include materials like hairs, and fecal samples (non-invasive samples) to ensure that these challenging materials, which is also the most frequently used sample materials in conservation genetics, may be properly analyzed using a validated protocol.

2. Materials and Methods

2.1 Materials

Fecal and hair samples were obtained during monitoring programs, and tissue samples were from legally shot bears during 2006-2012. Samples and sampling procedures have been previously described elsewhere [6, 8, 10, 11, 30]. Samples were analyzed with a previously developed gender test [10] and those identified as from male brown bears were included in our study. A total of 455 samples of male brown bears were included from Norway (n=189), Sweden (n=96), Finland (n=95), northwest Russia (n=65), Romania (n=5) and Canada (n=5). Sample from a male black bear were collected in Alberta in Canada and two male polar bears were from Kolyma in Russia. A total of five samples from female bears collected from the same materials described above were included to test gender specificity of the Y-STRs. Duplicate samples known to be from same individuals of tissue and hair (n=10) or tissue and scats (n=10) were used to demonstrate that all analyses from different materials provided identical Y-haplotypes.

2.2 DNA extraction, PCR and Y-STR analysis

DNA was extracted from hair and tissue using Qiagen DNeasy Tissue kit (Qiagen) and from feces using Invitek Stool kit (Stratec), following the manufactures` instructions. Hair samples were stored in room temperature in paper envelopes, tissue in ethanol and feces in stool collection tubes with DNA stabilizer (Stratec). The yield of DNA in tissue from male bears, female bears and 12 other mammalian species (see chapter 2.3) was quantified using a NanoDrop 2000 (Thermo Scientific). Single PCRs were performed in a 10 µl containing 1x PCR Gold buffer (ABI), 200 µM dNTP (Eurogentec), 1.5 mM $MgCl_2$ (ABI), 0.5 µM of each primer (Life technologies), 1 U Amplitaq Gold DNA polymerase (ABI), 1x BSA (NEB) and 1 µl template DNA. All samples have been typed with success using 1 µl template in autosomal markers (lower threshold for successful typing 0.6 ng).

DNA amplification was on an ABI 2720 for 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 ended with final extension for 45 min at 72 °C. Multiplex-PCR development involved tests of different combinations of markers, primer concentrations and DNA materials (details not shown). For the final analysis, the 9 Y-STR were split into one pentaplex (A) and one tetraplex (B) (Table 1) in touchdown PCR-approach in 10 µl reaction volumes using the following conditions: 5 µl 2x multiplex PCR master mix (Qiagen Multiplex kit), 0.05 µg/µl BSA (NEB), adjusted primer set concentration (Table 1). PCR conditions on an ABI2720 were 95 °C for 10 min followed by 10 cycles of 94 °C for 30 s, 69 °C (decreasing by 1 ° C per cycle) for 30 s and 72 °C for 60 s. This was followed by 20 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s. The final step was conducted for 45 min at 72 °C.

PCR products (1 µl) were mixed with Genescan 500 LIZ (Applied Biosystems) size standard (0.25 µl) and Hi-Di formamide (9.75 µl) following capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The POP-7™ Polymer was used as separation matrix and the sample injection time were set to 8 s/2kv. PCR fragments were analyzed in GeneMapper 4.1 (Applied Biosystems). Prior to PCR, all samples were verified to contain bear DNA as described in [6]. We used 600 RFU as lower threshold for including results from any of the Y-STRs in the Y-haplotypes produced from multiplex PCRs. To check for possible contamination, negative controls were included for every 7th sample in all measurements in this study.

2.3 Testing of PCR specificity, sensitivity and precision

To test for species specificity in the two multiplex PCR reactions (A and B), we used approximately 1 ng template DNA from 12 other mammalian species; 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*), badger (*Meles meles*) raccoon dog (*Nyctereutes procyonoides),* dog (*Canis familiaris)* and human. We used 1 ng female template DNA from brown bears (*Ursus arctos*), Canadian black bear (*U. americanus*) and polar bears (*U. maritimus*) to analyze gender specificity.

Two positive controls (tissue) of male bear DNA (1 ng template DNA) were included for species specificity and gender specificity. Sensitivity was evaluated by PCR amplification of DNA from male brown bear muscle tissue in the range 20–0.1 ng. Measurements of within-run precision and stutter ratios were performed in 10 independent amplifications and subsequent runs of a single sample of feces, hair and tissue, respectively.

2.4 DNA Sequencing

The tandem repeat array and the immediate upstream and downstream sequences at each of the nine loci were analyzed by DNA sequencing. PCR products amplified from DNA from male brown bears were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) as recommended by the manufacturer. Single PCR-primers from Table 1 were used as sequencing primers in forward and reverse sequencing reactions, respectively. Forward and reverse sequences from each sample were aligned in Sequencher 4.7. The allelic sequences from each locus were aligned and the sequence and size variation at each locus was determined by manual inspection.

3. Results

3.1 Tests of species and gender specificity and multiplex PCR combinations.

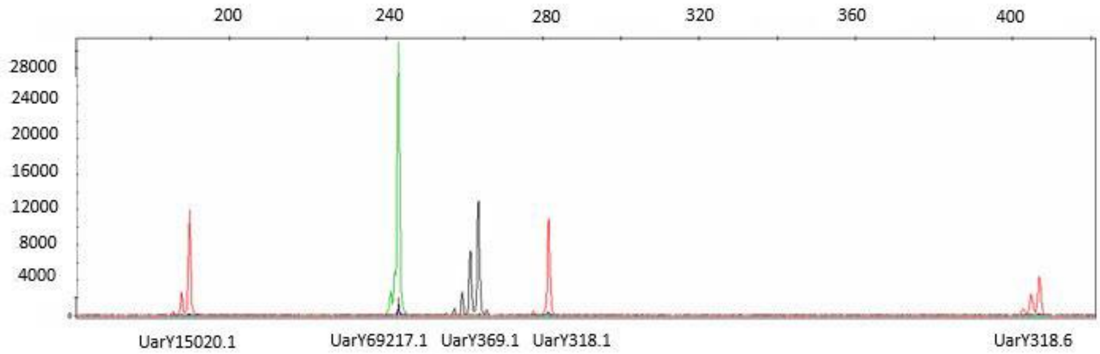
Template DNA from one black bear and two polar bears was tested in the different multiplex combinations of the nine Y-STRs using the primers described in methods. We found that all nine markers were amplified for both species. The allele-sizes observed in one black bear and two polar bears were within the allele-size range found in our brown bear populations, indicating that alleles from these markers are not grouped into discrete allele sizes among different bear species. This would also be consistent with findings in Bidon et al [29].

The specificity of the primers to bear species was confirmed using template DNA from males of 12 other mammalian species (see chapter 2.3). Our result showed that all species gave a negative result in multiplex reactions tested.

Similarly, we could not detect any PCR products in multiplex reactions tested when we used DNA from five female bears as template.

Different combinations of markers, primer concentration and DNA- material from male brown bears was tested in multiplex PCR reactions (data not shown). The highest signal to noise ratio was achieved when the nine Y-STR were split into one pentaplex (A) and one tetraplex (B) PCR reaction. Figure 1 demonstrate results obtained using these two multiplex PCRs. The combination of markers and primer concentrations used in multiplex reaction A and B are given in Table 1.

Multiplex A



Multiplex B

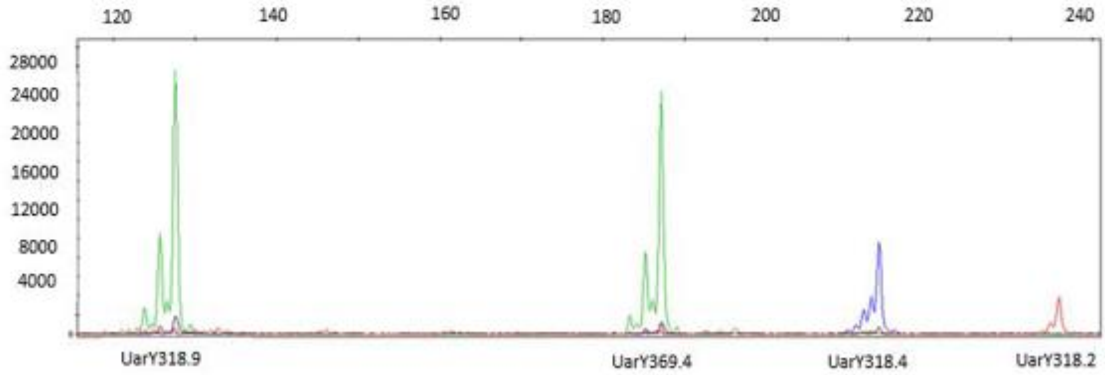


Figure 1. Chromatograms from capillary electrophoresis (ABI 3130XL) showing multiplex PCR reactions A and B (see also Table 1) for the nine brown bear Y-chromosomal STRs. Template DNA (1 ng) were from brown bear tissues. The names of the STR-markers are indicated below the peaks, and the peak height (RFU) is indicated on the Y-axis.

Table 1: PCR primers, alleles and multiplex PCR set-up for nine Y-STRs from brown bears

Locus	Primer sequences (5`-3`)[a]	Repeat motif	Allele size range (bp)[b]	No. of alleles observed[c]	PCR multiplex	Primer conc., Dye
UarY318.4	F:TACCTGGCTGGCTTTCTTGG R:CACTGTTGGTTTTGGCTCCG	GA	213-215	2	B	1 µM,FAM
UarY318.2	F:CAGGCTGACACTGGGGATTT R:AAGAGGGAGTCATCTGGGGT	TA	233-235	2	B	3 µM, PET
UarY318.9	F:CACTCAGGCACCCCTCTATC R:TGGCCAGGATACAGAAACAAC	AC	127-131	3	B	1 µM, VIC
UarY369.4	F:AGGCATCCATTCTATCACCAC R:TGTGGATGTATCTGCCCAAC	AC	182-200	7	B	1 µM, VIC
UarY318.1	F:GGGATCAAGCCCCACATCAA R:ACTTGTAGATGCACATCTGTGGT	AAAT	281-289	3	A	2 µM, PET
UarY69217.1	F:CTCCACCTTGTCTGCCACTC R:TTCCCCTCCCTTTCTGTCCT	TG	243	1	A	1 µM, VIC
UarY318.6	F:GCTGGCTGTCTCTCTCTCTGA R:AAATTCCTTTGGAAACGTCCT	TG	400-410	6	A	3 µM, PET
UarY15020.1	F:TGCAATTTCTCTCAAACAACTTCCT R:GCGATGAAGGTCAGAGCAGT	TG	185-189	3	A	1 µM, PET
UarY369.1	F:TCCCTGAATGAGCAGTAGCC R:GGGGTATTGCGTTGCATTGG	TG	249-273	9	A	2 µM,NED

[a] F forward, R reverse, from Bidon et al. 2014

[b] Allele size range in base pairs observed in 455 male brown bears (see Table 4)

[c] A total of 36 different alleles were observed among 455 male brown bears (see Table 4).

3.2 Sensitivity, precision and stutter ratios

We tested a concentration series of 20, 10, 1, 0.5, 0.2, 0.1, 0.05, 0.04, 0.03, 0.02 and 0.01 ng of template DNA in the two multiplex reactions. All markers in multiplex A were successfully typed with signals above the lower peak height threshold of 600 RFU with template DNA in the range 20–0.1 ng while multiplex B showed successful typing of all markers with template DNA in the range 20-0.2 ng. All samples representing duplicates from same individual, but template extracted from different materials, showed identical results for all parallel samples.

We also tested the within-run precision and stutter ratios using ten independent amplifications and subsequent runs of one sample of feces, hair and tissue (Table 2). These results show that the standard deviations (S.D.) from allele length measurements of all the nine loci tested were between 0.05 bp and 0.14 bp. Within-run precision and stutter ratios showed very similar results for hair-, fecal- and tissue-samples (Table 2). Stutters at -1R was observed in all markers (Figure 1). We found that these stutter ratios ranged from 0.04 to 0.63 from all materials (Table 2). Stutter in position -2R at loci UarY369.1, UarY318.4, UarY318, UarY369.4 and UarY318.9 was observed, but at much lower proportions (<0.04) than stutter band at -1R. Stutter one repeat larger than the true allele (+1R) was also observed, but in very small proportion (<0.05).

Table 2: Measurements of precision and stutter ratio for Y-chromosomal STRs from brown bears.

Locus[a]		Allele/genotype[a]	Mean (bp)[b]	Stutter ratio[c]
UarY318.4	Feces	213	213.76 (0.06)	0.21 (0.23)
	Hair	213	213.71 (0.05)	0.23 (0.24)
	Tissue	213	213.72 (0.07)	0.24 (0.31)
UarY318.2	Feces	235	235.78 (0.14)	0.10 (0.11)
	Hair	235	235.66 (0.14)	0.10 (0.60)
	Tissue	235	235.76 (0.09)	0.10 (0.10)
UarY318.9	Feces	127	127.59 (0.14)	0.33 (0.35)
	Hair	125	125.66 (0.10)	0.24 (0.26)
	Tissue	127	127.51 (0.11)	0.33 (0.35)
UarY369.4	Feces	192	192.82 (0.13)	0.40 (0.43)
	Hair	184	185.12 (0.13)	0.20 (0.22)
	Tissue	186	187.04 (0.13)	0.29 (0.36)
UarY318.1	Feces	281	281.51 (0.10)	0
	Hair	285	285.38 (0.09)	0.04 (0.05)
	Tissue	285	285.40 (0.07)	0.04 (0.05)
UarY69217.1	Feces	243	243.13 (0.08)	0.08 (0.11)
	Hair	243	243.08 (0.05)	0.12 (0.15)
	Tissue	243	242.88 (0.07)	0.18 (0.23)
UarY318.6	Feces	406	406.87 (0.13)	0.50 (0,58)
	Hair	404	404.74 (0.08)	0.49 (0.52)
	Tissue	404	404.71 (0.07)	0.49 (0.50)
UarY15020.1	Feces	189	189.56 (0.11)	0.23 (0.28)
	Hair	187	187.52 (0.08)	0.20 (0.20)
	Tissue	187	187.61 (0.11)	0.21 (0.29)
UarY369.1	Feces	263	263.58 (0.08)	0.55 (0.61)
	Hair	267	267.67 (0.05)	0.63 (0.64)
	Tissue	267	267.69 (0.04)	0.62 (0.63)

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

[a] Genotype nomenclature is based on PCR fragment sizes.

[b] Mean value allele sizes when measured with POP7 on ABI3730, with SD from in within-run measurement of 10 run per sample.

[c] Median stutter ratio with upper 95% percentile in parenthesis.

3.3 Investigation of repeat structure and gene variation for the nine Y-STRs

DNA sequencing was performed on the largest and the smallest alleles observed for each marker. The sequencing revealed that all size variation observed between the two alleles selected from same loci could be explained by variation in repeat numbers in the tandem repeat arrays (Table 3). Eight loci were tandem arrays of dinucleotide repeats while one had a repeat array of tetranucletide repeats (UarY318.1). Seven of the loci showed simple tandem repeat array structures while loci UarY318.2 and UarY69217.1 showed compound repeat structures (Table 3).

Table 3. DNA sequencing of tandem repeat structures of nine Y-chromosomal STRs from brown bears

Locus	Allele[a]	No. repeats	Repeat structure
UarY318.4	213	12R	(GA)$_{12}$
	215	13R	(GA)$_{13}$
UarY15020.1	187	11R	(GT)^{11}
	189	12R	(GT)$_{12}$
UarY318.1	281	9R	(AAAT)^{9}
	289	11R	(AAAT)$_{11}$
UarY318.2	235	18R	(TA)^{4}(TG)(TA)^{2}(TG)(TA)(T)(TA)^{7}(A)(TA)^{4}
Uar318.9	127	14R	AC^{14}
	131	16R	AC^{16}
UarY369.1	259	18R	*GT*^{18}
	273	25R	*GT*^{25}
UarY369.4	186	15R	(AC)$_{15}$
	200	22R	(AC)^{22}
UarY69217.1[b]	243	11R	(TG)^{6}(TA)(TG)^{5}
UarY318.6	400	15R	(TG)^{20}
	410	22R	(TG)^{25}

[a] Nomenclature of alleles is based on PCR fragment size.
[b] Monomorphic in this study.

Allele size measurements showed that there was allelic variation in eight of the nine Y-STRs analyzed in our material. The number of alleles observed in each marker was from 2-9. We found 36 different alleles among the 453 males analyzed. Allele frequencies for each locus are given for each country in Table 4. Rare alleles represented by only one individual were alleles 249, 273 (UarY369.1) and 410 (UarY318.6). In addition, 10 other alleles were present at low frequencies (>0.05). Locus UarY69217.1 did not reveal any size variation (monomorphic). Locus UarY318.2 showed allele size variations in individuals from Canada and Romania (allele 233) while all bears from Northern Europe were identical (allele 235). A total of 45 different Y-haplotypes were revealed in our material (Table 5). Some Y-haplotypes are fairly common among brown bears included in our material (mostly representing northern Europe) with frequencies ranging from 0.2 to 98.2 % in polymorphic markers. However, 19 different Y-haplotypes were observed in single individuals only.

Table 4: Allele frequencies for nine Y-STRs for brown bears marker per country. Frequencies are first given per country of origin of samples and then for the total sample. The number of individuals analyzed for each country and in total, as well as the number of individuals carrying the respective allele are given in brackets.

		Allele frequency						
Marker	**Allele**	**Finland (95)**	**Norway (189)**	**Russia (65)**	**Sweden (96)**	**Romania (5)**	**Canada (5)**	**Total (455)**
UarY318.4	**213**	0.842 (80)	0.899 (170)	0.846 (55)	0.846 (96)	1.000 (5)	1.000 (5)	0.903 (411)
	215	0.161 (15)	0.101 (19)	0.154 (10)	-	-	-	0.097 (44)
UarY369.1	**249**	-	-	-	-	0.200 (1)	-	0.002 (1)
	259	0.022 (2)	-	0.031 (2)	-	0.200 (1)	-	0.011 (5)
	261	0.043 (4)	0.333 (63)	0.015 (1)	0.427 (41)	-	-	0.241 (109)
	263	0.043 (4)	0.106 (20)	0.138 (9)	0.031 (3)	-	-	0.079 (36)
	265	0.527 (49)	0.312 (59)	0.385 (25)	0.313 (30)	0.200 (1)	-	0.362 (164)
	267	0.232 (22)	0.196 (37)	0.215 (14)	0.229 (22)	0.200 (1)	-	0.211 (96)
	269	0.054 (5)	0.005 (1)	0.092 (6)	-	0.200 (1)	1.000 (5)	0.040 (18)
	271	0.086 (8)	0.048 (9)	0.123 (8)	-	-	-	0.055 (25)
	273	0.011 (1)	-	-	-	-	-	0.002 (1)
UarY15020.1	**185**	-	-	-	-	-	1.000 (5)	0.011 (5)
	187	0.568 (54)	0.376 (71)	0.585 (38)	0.585 (39)	-	-	0.407 (185)
	189	0.441 (41)	0.624 (118)	0.415 (27)	0.771 (74)	1.000 (5)	-	0.585 (265)
UarY318.2	**233**	-	-	-	-	0.600 (3)	1.000 (5)	0.018 (8)
	235	1.000 (95)	1.000 (189)	1.000 (65)	1.000 (96)	0.400 (2)	-	0.982 (447)
UarY318.1	**281**	0.484 (45)	0.619 (117)	0.508 (33)	0.771 (74)	0.800 (4)	1.000 (5)	0.614 (278)
	285	0.505 (48)	0.381 (72)	0.492 (32)	0.229 (22)	0.200 (1)	-	0.385 (175)
	289	0.022 (2)	-	-	-	-	-	0.004 (2)
UarY369.4	**186**	0.337 (32)	0.265 (50)	0.308 (20)	0.229 (22)	-	-	0.273 (124)
	190	0.108 (10)	0.159 (30)	0.062 (4)	0.313 (30)	-	-	0.163 (74)
	192	0.269 (25)	0.545 (103)	0.338 (22)	0.458 (44)	0.200 (1)	1.000 (5)	0.442 (200)
	194	0.032 (3)	-	0.031 (2)	-	-	-	0.011 (5)
	196	0.204 (19)	0.032 (6)	0.154 (10)	-	0.200 (1)	-	0.079 (36)
	198	0.032 (3)	-	0.077 (5)	-	0.600 (3)	-	0.024 (11)
	200	0.032 (3)	-	0.031 (2)	-	-	-	0.011 (5)
UarY69217.1	**243**	1.000 (95)	1.000 (189)	1.000 (65)	1.000 (96)	1.000 (5)	1.000 (5)	1.000 (455)
UarY318.6	**400**	0.172 (16)	0.111 (21)	0.215 (14)	-	-	-	0.113 (51)
	402	0.258 (24)	0.032 (6)	0.246 (16)	-	-	-	0.102 (46)
	404	0.284 (27)	0.233 (44)	0.323 (21)	0.229 (22)	-	-	0.251 (114)
	406	0.280 (26)	0.608 (115)	0.169 (11)	0.771 (74)	1.000 (5)	1.000 (5)	0.521 (236)
	408	0.011 (1)	0.016 (3)	0.046 (3)	-	-	-	0.015 (7)
	410	0.011 (1)						0.002 (1)
UarY318.9	**127**	0.684 (65)	0.413 (78)	0.646 (42)	0.260 (25)	0.800 (4)	1.000 (5)	0.481 (219)
	129	0.062 (6)	0.005 (1)	0.138 (9)	-	0.200 (1)	-	0.038 (17)
	131	0.258 (24)	0.582 (110)	0.215 (14)	0.740 (71)	-	-	0.483 (219)

Table 5: Frequencies of brown bear Y-chromosome haplotypes* per country. Frequencies are first given per country of origin of samples and then for the total sample. The number of individuals analyzed for each country and in total, as well as the number of individuals carrying the respective haplotype are given in brackets.

	Haplotype frequency						
Haplotype	**Finland (95)**	**Norway (189)**	**Russia (65)**	**Sweden (96)**	**Romania (5)**	**Canada (5)**	**Total (455)**
1.01	0.137 (13)	0.101 (19)	0.092 (6)	-	-	-	0.084 (38)
1.13	0.011 (1)	-	-	-	-	-	0.002 (1)
1.19	-	-	0.015 (1)	-	-	-	0.002 (1)
1.23	0.011 (1)	-	0.046 (3)	-	-	-	0.009 (4)
1.25	-	-	0.031 (2)	-	-	-	0.004 (2)
1.37	0.011 (1)	-	-	-	-	-	0.002 (1)
2.02	0.021 (2)	0.101 (19)	0.077 (5)	0.031 (3)	-	-	0.064 (29)
2.05	0.063 (6)	0.148 (28)	0.031 (2)	0.313 (30)	-	-	0.147 (66)
2.06	0.021 (2)	0.005 (1)	0.046 (3)	-	-	-	0.013 (6)
2.07	0.189 (18)	0.026 (5)	0.123 (8)	-	-	-	0.069 (31)
2.08	0.021 (2)	0.333 (63)	-	0.427 (41)	-	-	0.236 (106)
2.10	-	-	0.031 (2)	-	-	-	0.004 (2)
2.11	0.021 (2)	-	0.015 (1)	-	-	-	0.007 (3)
2.12	0.021 (2)	-	0.031 (2)	-	-	-	0.009 (4)
2.14	0.032 (3)	-	0.031 (2)	-	-	-	0.011 (5)
2.16	-	-	0.015 (1)	-	-	-	0.002 (1)
2.17	-	-	0.031 (2)	-	-	-	0.004 (2)
2.18	-	-	0.015 (1)	-	-	-	0.002 (1)
2.22	0.021 (2)	-	-	-	-	-	0.004 (2)
2.24	-	0.005 (1)	-	-	-	-	0.002 (1)
2.27	0.011 (1)	-	-	-	-	-	0.002 (1)
2.28	-	0.005 (1)	-	-	-	-	0.002 (1)
2.29	-	-	0.015 (1)	-	-	-	0.002 (1)
2.30	-	-	0.015 (1)	-	-	-	0.002 (1)
2.31	0.032 (3)	-	-	-	-	-	0.007 (3)
2.32	0.011 (1)	-	-	-	-	-	0.002 (1)
3.03	0.074 (7)	0.016 (3)	0.015 (1)	-	-	-	0.024 (11)
3.04	0.021 (2)	0.048 (9)	0.077 (5)	-	-	-	0.036 (16)
3.09	0.179 (17)	0.185 (35)	0.123 (8)	0.229 (22)	-	-	0.182 (82)
3.15	-	-	0.031 (2)	-	-	-	0.004 (2)
3.20	0.011 (1)	0.011 (2)	0.031 (2)	-	-	-	0.011 (5)
3.21	0.011 (1)	0.016 (3)	0.031 (2)	-	-	-	0.013 (6)
3.26	-	-	0.015 (1)	-	-	-	0.002 (1)
3.33	-	-	0.015 (1)	-	-	-	0.002 (1)
3.34	0.011 (1)	-	-	-	-	-	0.002 (1)
3.35	0.021 (2)	-	-	-	-	-	0.004 (2)
3.36	0.032 (3)	-	-	-	-	-	0.007(3)
3.38	0.011 (1)	-	-	-	-	-	0.002 (1)
x.39	-	-	-	-	0.200 (1)	-	0.002 (1)
x.40	-	-	-	-	0.400 (2)	-	0.004 (2)

x.41	-	-	-	-	0.200 (1)	-	0.002 (1)
x.42	-	-	-	-	0.200 (1)	-	0.002 (1)
x.43	-	-	-	-	0.200 (1)	-	0.002 (1)
x.44	-	-	-	-	-	1.000 (5)	0.011 (5)

* Haplotypes are based on variation in 8 Y-STRs (see table 4). For haplogroups 1, 2 and 3, see [33], x= haplogroup not determined.

4. Discussion

In this study, we have validated nine Y-chromosomal STRs for use in conservation genetics and wildlife forensic. We find that a dual multiplex PCR approach represents a fast and precise assay for Y-chromosomal DNA profiling and haplotyping of hair- , fecal- and tissue samples from brown bears. All nine Y-STRs used for this work had originally been identified and aligned in genomic sequence data from polar and brown bears, and also tested successfully on American black bears [29]. We recently showed that the patterns of PCR fragment sizes (allele sizes) differ for the three bear species on a phylogeographic scale [29]. Additionally, we here report a large and geographically extensive set of population data for the brown bear, while detailed intra-population assessments of Y-chromosomal variation are still lacking for polar bears and American black bears.

To test for bear specificity we used test species that bears may predate upon, as well as hair and fecal samples from other carnivores that may erroneously be collected as bear samples in the field. We also included human DNA to assure that any contamination by handling samples would not produce any false results. Even though DNA from more species may have been included in the test, we may conclude that all these nine Y-markers show very strong male bear-specific amplification. The negative results from female bear DNA (absence of amplification signals) from all three species show that all nine Y-STRs are male-specific sequences that do not have any close homologues on the X-chromosome or autosomes. This finding is also supported by our DNA sequencing results on the alleles, i.e no indication of heterozygosity that may be a result of a second copy interfering with the assay.

Sensitivity testing showed that the Y-specific multiplex PCR method worked well down to amounts of 0.2 ng template DNA. This is more sensitive than for autosomal STRs on similar samples that are successfully amplified down to 0.6 ng, but less sensitive than our previous results on multiplex PCR assays for sex determination at DNA template levels as low as 0.02 ng [31]. The latter is based on very small amplicon sizes (100-160bp) from Y- and X-chromosomes, while the PCR fragments for this study have a wider range (127-410 bp). Thus, reducing the larger Y-STR amplicon sizes and then repeating the multiplex development may further improve the sensitivity of our novel assay. However, this may also be unfavorable because of Y-sequence structures and motifs that are not ideal for PCR priming (results not shown).

Precision was in general high for both multiplex assays A and B, also when compared to autosomal STRs (see [6]). Stutter ratios were as expected very low for the single tetranuclotide repeat (UarY318.1), and more pronounced for the remaining eight dinucleotide repeats. Stutter ratios were in general much lower than measured previously for brown bear autosomal STRs [6]. We tested template DNA extracted from hairs, scats and tissues in the multiplex assays. All replicated sample materials from the same individuals showed identical Y-haplotypes and very low variation in precision and stutter ratios were observed. This justifies that larger monitoring or conservation studies may combine Y-haplotypes generated from the three sample materials tested in our validated multiplex assays.

Sequencing of alleles showed that the size variation observed could be explained as depending on the number of repeats. A nomenclature of alleles based on the number of repeats instead of PCR fragment size used in human forensics is also recommended for STR markers applied for wildlife forensics [32]. Sequencing of all alleles observed could facilitate such a nomenclature on alleles in the Y-STRs validated in our study. Independent of this, we offer sample DNA of alleles sequenced in our study to other laboratories for use as inter-laboratory calibrators.

Allele frequencies for 36 different alleles showed only three different alleles that were present in only one male, while 10 additional alleles were found to be rare in Northern Europe. Including ten samples from other regions (Canada and Romania) only added one extra allele, but showed that the corresponding marker (UarY318.2) also is polymorphic in brown bears. Thus even if marker UarY69217.1 was monomorphic in Northern Europe, we still suggest that this marker be kept in the multiplex, for application of our method in other bear populations and species.

In wildlife forensics and illegal trade with endangered species, determination of population and geographical origin of an unknown forensic sample can contribute to resolve cases [23, 34, 35], but this requires the source population to be sufficiently genetically distinct from other candidate populations, as well as large reference data [26]. Previous studies [29] and our present results show region-specific Y-haplotype frequencies across Europe as well as some region-specific haplotypes [33]. This indicates that the construction of a Y-STR-based profiling system, especially in combination with one based on autosomal STRs [6, 30], may contribute significantly to resolving geographical origin in brown bear forensic cases in the future.

Resolving wildlife forensic cases like poaching, illegal killing, collection and trade is most commonly achieved using autosomal STR-profiling and mtDNA-sequencing [27]. The use of Y-STR markers provides additional information and is already commonly used in human forensics cases to resolve male-female and male-male mixtures, e.g., to reliably determine the minimum number of male individuals presumable involved [22]. Our Y-STR marker developed for brown bear could provide same useful data in wildlife forensic cases as well as in predation events [28]. Another argument for the promise of Y-markers in bears is the strong male-biased dispersal, which means that males are at larger risk of being victims of poaching, or being involved in conflict with humans.

5. Conclusion

Our work is focused on validation of the Y-profile system for brown bears. In our opinion, such a validation is a pre-requisite for its use in forensics, and such a standard is also desirable in conservation genetics. To use this Y-profile system for geographical assignment there need to be developed an even larger population database eventually containing bears from all over the world. If the research communities use the Y-profile system presented here, such data may be compared and thus our Y-system represents a means to achieve this rather than a proof that our Y-system may be used in such a manner.

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