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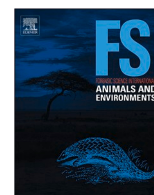

Characterizing North Carolina black bear (*Ursus americanus*) populations using UrsaPlex v2.0

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ABSTRACT

American black bears (*Ursus americanus*) have been successfully restored in North Carolina (NC) due to management and research efforts by the North Carolina Wildlife Resources Commission and various partners. Investigations by law enforcement in NC involving black bears typically center on a) illegal take, b) purchase, possession, and sale of bear parts, or c) human-bear interactions. Effective prosecution of illegal parties requires methods that permit individual identification of black bears. Typically, individualization is achieved using robust DNA profiling and comparison to accepted genetic databases. Currently, a genetic database of highly variable loci genotyped from black bears across their range in NC does not exist. To address this, we focused on genotyping NC black bears using an updated version of UrsaPlex (UrsaPlex v2.0), a short tandem repeat (STR) panel of 11 loci and three sex-linked markers, previously developed to permit individualization for California black bears. Biological samples were obtained from NC black bears following IACUC approved protocols. Total genomic DNA was isolated from each sample and genotyped using UrsaPlex v2.0. The number of alleles in the final genotyped population (n = 173) ranged from five (UamD118 and UamB8) to 33 (UamA9). The observed heterozygosity (H_o) ranged from 0.471 (UamB1) to 0.872 (UamA9), while the expected heterozygosity (H_e) ranged from 0.469 (UamB1) to 0.919 (UamA9). The probability of identity (PID) was calculated as 6.9×10^{-13}. These results demonstrate the potential utility of UrsaPlex v2.0 for permitting individual identification of NC black bears for use in forensic casework and for population management.

1. Introduction

American black bears (*Ursus americanus*) were abundant in North Carolina (NC) when European settlers first arrived, but expanding settlement, unregulated harvest, and habitat changes during the 18th and 19th centuries caused populations to decline to less than 1,000 bears by the 1970's (located only in remote areas of the mountains and the coastal plains) [1]. In 1981, the North Carolina Wildlife Resources Commission (NCWRC) developed the first Black Bear Management Plan. Through a combination of hunting regulations, research, habitat conservation improvements, and the creation of designated bear management areas to protect breeding females, the NC population increased to ~20,000 individuals statewide by 2022 [1].

Following the restoration of black bears in NC, law enforcement investigations involving black bears have increased. There are three main scenarios in which black bears are involved in investigations in NC and across the United States, a) illegal take, primarily out of season harvest or harvesting more than one bear per season, b) purchase, possession, and sale of bear parts (most commonly gallbladders and paws) for purported medicinal purposes, or c) human-bear interactions due to increased urbanization and recreational activities encroaching into black bear habitats [2] (*pers. comms.*). In all scenarios, determining the individual animal is essential, whether this is to link a suspected poacher to the black bear carcass, or to ensure the correct bear is euthanized in

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Table 1
Summary UrsaPlex v2.0 information for North Carolina black bears: Locus name, primer sequence (lowercase letters represent sequence with no homology to template DNA, whereas homologous regions are shown in uppercase), dye of the forward primer, final concentration of forward and reverse primer in 10 μL amplification reactions, and allele size range (base pairs). For all NC black bears (n = 173) and separately for Western (n = 116) and Eastern (n = 57) black bears, the number of alleles, heterozygosity observed and expected (H_o and H_e), and polymorphic information content (PIC) is provided.

Locus Name	Primer Sequence (5' – 3')	Dye	UrsaPlex v2.0 Final Primer Concentration (μM, F and R)	Approximate Size Range (bp)	NC Combined					Western					Eastern				
					# Alleles	# of bears	H_o	H_e	PIC	# Alleles	# of bears	H_o	H_e	PIC	# Alleles	# of bears	H_o	H_e	PIC
UamD1a	F: GGCGGGAGAGCATATTTC; R: gtttcttTTGGGGTGGAGCCTACTT	6-FAM	0.06	113–137	7	173	0.630	0.697	0.645	5	116	0.603	0.683	0.626	6	57	0.684	0.713	0.668
UamD118	F: TGGGTTTGGCATTTTTATC; R: gtttcttCAGAGCACCACACTGATACTC	6-FAM	0.175	176–192	5	173	0.636	0.667	0.612	4	116	0.629	0.649	0.581	5	57	0.649	0.663	0.620
UamA9[a]	F: TCCTTCCTACTTCTCTCTCTCC; R: gtttcttCCTCTCATGCCCTCACTC	6-FAM/PET[d]	1	176–316	33	172	0.872	0.919	0.914	20	115	0.878	0.868	0.856	25	57	0.860	0.917	0.913
UamA107	F: ATTCCCATTGGTGCCTCT; R: gtttcttCCCCCATCAAAAATCCAT	VIC	0.1	154–190	11	173	0.757	0.835	0.814	11	116	0.784	0.821	0.798	8	57	0.702	0.773	0.750
UamB1	F: GGCACCAATGTTACTTTCCTAC; R: gtttcttGTGGGTGGAGAGAAGTTTAGAA	VIC	0.33	238–258	6	172	0.471	0.469	0.442	5	116	0.440	0.413	0.385	5	56	0.536	0.543	0.490
UamB8	F: CATACCTGTGGCTGAATCTAG; R: gtttcttAGCACTCAGGATAGTTTCACTC	VIC	1	281–297	5	173	0.561	0.608	0.550	5	116	0.595	0.593	0.536	5	57	0.491	0.632	0.570
UamD112	F: GAATCCTCTCCAAGACCTATG; R: gtttcttGTTTTCCTTATCCCTGAACTG	NED	0.1	121–199	21	173	0.855	0.909	0.902	17	116	0.888	0.885	0.874	16	57	0.789	0.873	0.860
UamA2[b]	F: CCAGCTCCTTTAACACTGCTC; R: gtttcttgtttcttgtttcttCATGGGCTTTTGTTCAGTACC	NED	0.2	232–257	7	173	0.757	0.824	0.800	7	116	0.698	0.806	0.778	7	57	0.877	0.828	0.806
UamD116[a,c]	F: ATTCCCCAAACACAGACCAC R: gtttcttACCTCTCACCCTGTTTGTG	NED	0.28	282–321	11	173	0.694	0.792	0.770	10	116	0.672	0.712	0.678	11	57	0.737	0.870	0.856
UamD103	F: AGCCTTATCAGTTAGGGTTTTC; R: gtttcttCTGGCTTTCAGACTGGAAC	PET	0.1	150–173	7	173	0.618	0.634	0.567	5	116	0.603	0.587	0.503	6	57	0.649	0.694	0.642
UamB125	F: ACCAGCCTGAGGTATTGC; R: gtttcttATGGATGGTGGATGGATC	PET	1.2	239–309	11	169	0.751	0.791	0.761	9	114	0.781	0.802	0.774	10	55	0.691	0.580	0.559
BearSMCY	F: GTCTTCCTCCTTAGAGGGTAATTAGG; R: gtttcttTTCGTTTGATAATGGCCTAAAACTG	PET	0.7	106	1		N/A	N/A	N/A	1		N/A	N/A	N/A	1		N/A	N/A	N/A
BearZFX[a,c]	F: TGCAAAGAATCTGATTATGTTA R: gtttcttTCGCCACCCRCAAATAG	PET	0.13	113	1		N/A	N/A	N/A	1		N/A	N/A	N/A	1		N/A	N/A	N/A
Bear318.2	F: AAGAAAAGTCATGCAACAGATACAG; R: gtttcttTGATGCTTTGTGATCCTAATGTG	PET	0.09	129	1		N/A	N/A	N/A	1		N/A	N/A	N/A	1		N/A	N/A	N/A

[a] Forward primer has updated 5' dye label in UrsaPlex v2.0.
[b] Reverse primer has modified 5' end triple pigtail [3,30] in UrsaPlex v2.0.
[c] Novel forward primer sequence in UrsaPlex v2.0 (E. Meredith, unpublished data).
[d] Forward primer was reordered using PET dye for reworked samples.

the interest of public safety.

Short tandem repeats (STRs) are repeats of 2–8 nucleotides that occur directly adjacent to each other in the nuclear genome and are considered the gold standard for individualization in human forensics. Also, STRs have been characterized for wildlife species encountered in forensic casework (*e.g.,* [3–7]) and have been used to provide evidence for prosecution of crimes involving rhinoceros [8] and elephant [9]. These species-specific panels are typically developed as needed by wildlife forensic laboratories (not commercial companies) and used in scenarios including a) individual matching of a questioned item to an animal, b) determining the minimum number of animals present in evidence (to ensure harvest limits are not violated), c) relatedness of multiple individuals, or d) geographic origin of a sample [10]. In the United States there are only a very small number (<10) of forensic laboratories that perform STR genotyping for non-human biological evidence, and these rely exclusively on self-established allele frequency databases for downstream statistical calculations [10].

To address a need for black bear individual identification, the California Department of Fish and Wildlife – Wildlife Forensic Laboratory developed UrsaPlex; a multiplex panel targeting 11 STRs, supplemented with three additional loci for sex determination [2,11]. While UrsaPlex has been shown to permit individual identification of California black bears [2], its utility for individual identification of NC black bears was unknown. To address this information gap, this study focused on a) genotyping NC black bears using an updated version of UrsaPlex (UrsaPlex v2.0) across the extent of their range, and b) complete population statistics to establish whether the UrsaPlex v2.0 would be suitable for individual identification of NC black bears. Notably, this study did not focus nor will report on results from an internal validation of UrsaPlex v2.0 for use in forensic casework.

2. Materials and methods

2.1. Sample collection and DNA isolation

Following recommendations from the International Society of Forensic Genetics, a representative sample of > 200 NC black bears from across their known geographic range were acquired [12]. Archived serum (n = 54), tissue (n = 6), and whole blood (n = 233) samples from NC black bears were acquired from collections stored at North Carolina State University (NCSU; [13–15]). Black bear samples in these archived collections sourced from western NC localities (primarily Buncombe, Polk, Macon and Transylvania counties) were collected during 1999–2002 and 2014–2017, whereas those sourced from Eastern areas of the state (primarily Hyde, Tyrell, Camden, Washington and Curritick counties) were collected between 2003 and 2017. Additional fresh saliva swabs (n = 75) from black bears across the state were acquired under an existing IACUC protocol for live captures (NCSU; 19–723) and collected by NCWRC from deceased animals. DNA was isolated using either the DNeasy Blood & Tissue Kit (Qiagen) (tissue and saliva samples) or the QIAsymphony DSP DNA Mini Kit on the QIAsymphony SP extraction robot (blood and serum samples) following the manufacturer's specific protocols. Extracts were quantified with the Invitrogen™ Qubit™ Fluorometer (Thermo Fisher Scientific).

2.2. Modifications to UrsaPlex

Primer sequences and dye labels for several loci in the original UrsaPlex panel [2] were modified to address known overlap issues and create the updated UrsaPlex v2.0 panel [E. Meredith, unpub. data] (Table 1).

2.3. Amplification and genotyping with capillary electrophoresis

A total of 11 tetranucleotide STRs and three sex-markers (1 X-chromosome and 2 Y-chromosome) were targeted using the updated UrsaPlex v2.0 panel [[2]; E. Meredith, unpub. data]. Genomic DNA samples were amplified using 5 μL 2X Qiagen Multiplex Master Mix, 1 μL UrsaPlex v2.0 Primer mix (Table 1), up to 10 ng of DNA, and PCR-grade water up to the 10 μL reaction volume. Sample amplification reworks (due to either amplification failure or marker overlap within the 6-FAM dye channel) used two separate primer mixes/amplifications: a duplex (UamD118 incorporating the 6-FAM dye and UamA9 incorporating the PET dye) and an updated multiplex (the nine remaining STRs and three sex-markers). UrsaPlex v2.0 primer concentrations (Table 1) and other amplification reaction components were maintained for the duplex amplifications. PCR amplification was completed using a Veriti Thermocycler (Applied Biosystems) with the following conditions: 95 °C for 15 min; 27 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 60 s; final extension at 72 °C for 10 min and a final hold at 10 °C. Amplicons were prepared for genotyping by combining 9.15 μL of HiDi Formamide (Thermo Fisher Scientific), 0.35 μL of GeneScan 600 LIZ dye Size Standard v2.0 (Thermo Fisher Scientific), and 0.5 μL of PCR product. Samples were transported on ice to either Cornell University Institute of Biotechnology's Biotechnology Resource Center (Ithaca, NY) or the NCSU Genomic Sciences Laboratory, each using a 3730*xl* Genetic Analyzer (Applied Biosystems) and standard fragment analysis protocols for separation.

2.4. Data analysis

Results were analyzed using GeneMarker HID 2.9.0 (SoftGenetics LLC) using an analytical threshold utilized by the California Department of Fish and Wildlife – Wildlife Forensic Laboratory in forensic casework (100 relative fluorescence units [RFUs]) [2]. Allele sizing was performed using the GeneScan 600 LIZ dye Size Standard v2.0 (Thermo Fisher Scientific). In the absence of an available allelic ladder, a panel of allele-specific bins for UrsaPlex v2.0 was provided by the California Department of Fish and Wildlife – Wildlife Forensic Laboratory. Due to differing instrumentation, capillary length and polymer used, the panel was adjusted as necessary using a series of positive controls with known genotypes. Given none of the positive control samples contained UamA9 alleles within the size range of UamD118, assignments of the novel smaller UamA9 alleles (*i.e.,* those sizing at 176, 180, and 184 base pairs) were calculated based on allele size and base pair difference from known alleles (Supplemental Information). The multi-locus genotypes were visually inspected by at least two analysts before finalizing, and any mixtures or amplification failures were removed from the dataset.

2.5. Population statistics

To identify any primary familial relationships (*e.g.,* siblings, parent/child *etc.*) the complete set of genotypes for NC black bears was analyzed using Lynch & Ritland's estimator [16] in GeneAlEx [17]. For primary familial relationships with a value of 0.25 or higher [18], one individual was randomly selected for removal from such pairs. The remaining black bears were categorized into a single population (herein referred to as "NC") and also as two separate populations, based on collection location from either the mountains or coastal plains of the state (herein referred to as "Western" and "Eastern", respectively). These designations were used for determination of observed heterozygosity (H_o), expected heterozygosity (H_e), number of alleles, and allelic frequency using GeneA1Ex [17]. Estimates of probability of identity (PID) and probability of identity siblings (PID_{SIBS}) [19] were conducted exclusively on the single NC population using GeneA1Ex [17]. Eleven loci from the UrsaPlex v2.0 panel were included for this analysis, and the three sex-determining markers were excluded. The calculation of polymorphic information content (PIC) was performed using the PopGenUtils and Polysats packages [20,21].

To explore the presence of subpopulations between NC black bears, ParallelSTRUCTURE 2.3.4 [22] was run through CIPRES Science Gateway [23]. The analysis of the final NC black bear dataset (n = 173)

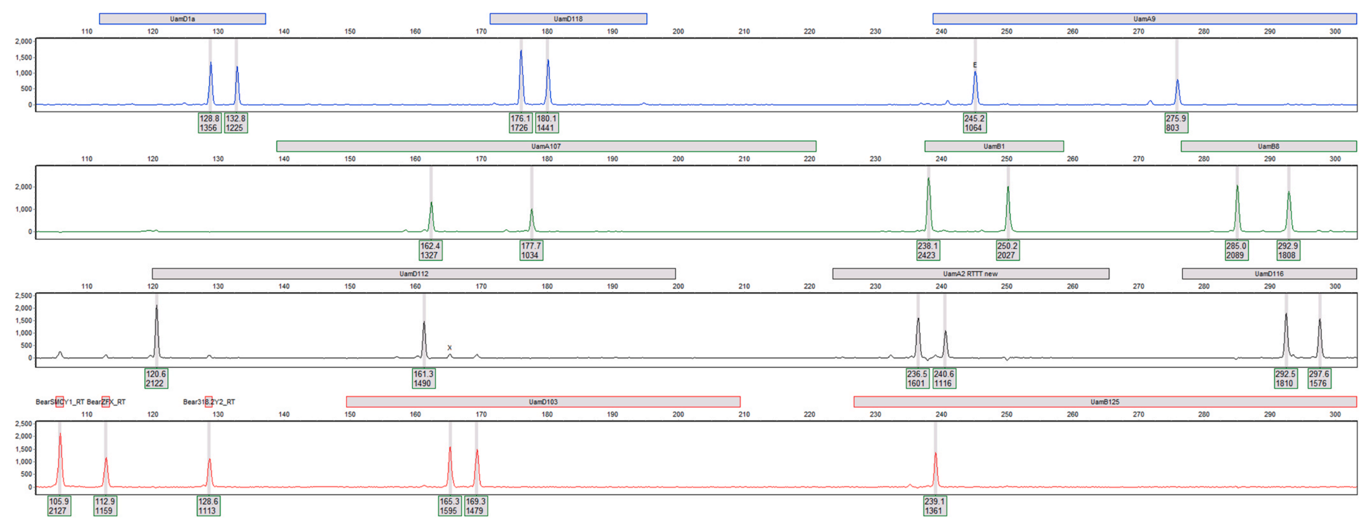

Fig. 1. Typical UrsaPlex v2.0 electropherogram (10 ng template, 27 cycles). Labels give (top to bottom) peak midpoint (base pair) and peak height (relative fluorescence units; RFUs).

based on 11 autosomal UrsaPlex v2.0 loci included parameters such as admixture, no prior population information (popflag = 0), correlated allele frequencies, and a burn-in period of 250,000 with 500,000 Markov chain Monte Carlo repetitions for 10 iterations (K = 1–6). The ParallelSTRUCTURE analysis was repeated with the addition of 47 voucher bear specimens from California [2] and included the 10 loci that overlap between the original and updated versions of the panel. The results from both analyses were analyzed using Evanno's Best K and the software packages pophelper v2.3.1, tidyr, dplyr, and ggplot2 [24–27]. Additionally, a Discriminant Analysis of Principal Components (DAPC) was performed using the ADEGENET software package in R for the final NC black bear dataset (n = 173) based on the 11 autosomal UrsaPlex v2.0 loci [28]. This method, in contrast to ParallelSTRUCTURE, relies on a non-model multivariate approach [29]. Twenty principal components were retained following cross-validation (xvalDapc). Potential sub-clustering within each original sample population (*i.e.,* Eastern and Western) was investigated as follows: (1) the optimal number of clusters was identified using a k-means clustering algorithm (find.clusters), (2) the Bayesian Information Criterion (BIC) was calculated for a range of K values, where K is the number of clusters and the optimal clustering value is determined by the lowest BIC, and (3) inferred clusters were used in an additional cross-validation analysis, where 10 principal components and three discriminant axes were retained.

3. Results and discussion

3.1. Performance of UrsaPlex v2.0

A total of 368 NC black bear samples were genotyped using UrsaPlex v2.0, with 97.6 % using the optimal 10 ng of DNA as input. Only 16 samples (4.3 %) were removed due to either being deemed a mixture of multiple individuals (*i.e.,* more than two alleles at any given locus), amplification failure, or unresolved artifacts. Notably, a complete profile was generated using as little as 1.1 ng DNA as input. A total of 352 samples had complete genotypes, however only 350 samples were carried forward for kinship analyses as two samples were obvious duplicates (*i.e.,* similar sample names with identical genotypes) and thus removed. Across all genotyped plates, the positive controls displayed the expected genotype and negative controls had no alleles above the analytical threshold. An example electropherogram for UrsaPlex v2.0 is shown in Fig. 1.

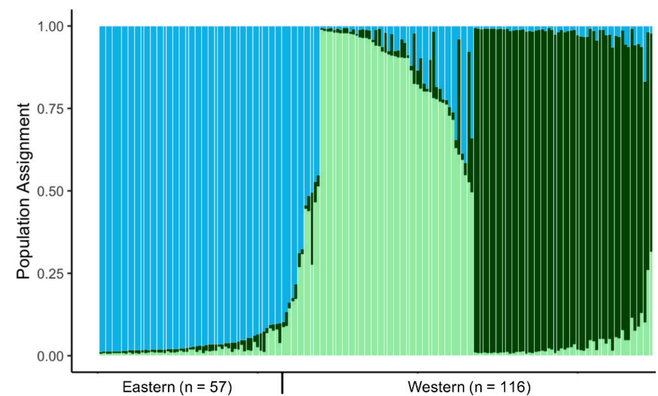

Fig. 2. Structure plot generated in Rstudio using ggplot2 package with the results from ParallelSTRUCTURE. Data plotted from all NC black bears (n = 173; displayed by collection location as Eastern or Western) inferred using 11 autosomal loci from UrsaPlex v2.0 at K= 3. The vertical bars along the x-axis each represent an individual black bear. The proportion of ancestry for each individual that can be attributed to one of the three inferred genetic clusters is shown by the three colors (light blue, light green or dark green) along the y-axis.

3.2. Kinship analysis and population statistics

Following the removal of first-degree relatives, a total of 173 individuals (49 % of complete genotypes) were remaining for population statistics analyses. For analyses completed with all individuals combined as a single population ("NC"), the average number of alleles represented in each locus was 11.3 (range = 5–33), mirroring the results reported for California black bears using the original UrsaPlex [2]. The average observed heterozygosity (H_o) was 0.691 (range = 0.471–0.872), lower than the average expected heterozygosity (H_e) of 0.740 (range = 0.469–0.919). The PID was 6.9×10^{-13}, while the PID_{SIBS} was 4.2×10^{-5}. Both PID and PID_{SIBS} values are similar to those calculated for California black bears using the original UrsaPlex [2]. Given previous studies have indicated that PID < 0.01 is sufficient for identifying individuals [19] and the NC black bear population is estimated at 20,000, these results show that UrsaPlex v2.0 is suitable for individual identification for forensic purposes (Table 1). When separated by collection location, Western NC black bears (n = 116) showed an average of 8.9 alleles per locus (range = 4–20), while the Eastern NC black bears (n = 57) showed an average of 9.5 alleles per locus (range = 5–25). The

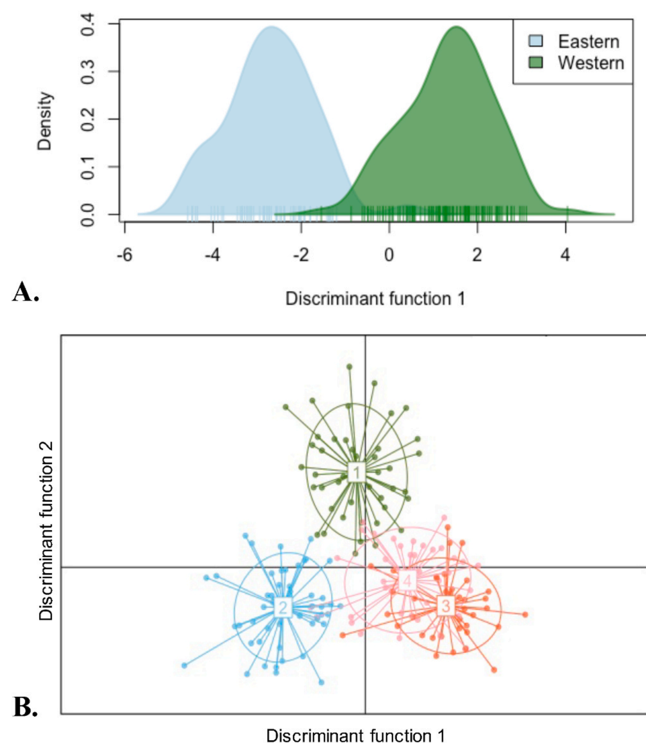

Fig. 3. Discriminant Analysis of Principal Components (DAPC) plots for all NC black bears (n = 173) inferred using 11 autosomal loci from UrsaPlex v2.0. (A) Differentiation between the two original populations (Eastern and Western) plotted across discriminant function 1 (twenty principal components were retained). (B) Scatter plot for the first two discriminant functions. Clusters numbered 1, 3, and 4 primarily include black bears collected from Western NC localities, whereas cluster numbered as 2 primarily includes black bears collected from Eastern NC localities.

average observed heterozygosity (H_o) was 0.735 (range = 0.440–0.888) and 0.697 (range = 0.491–0.877), for the Western and Eastern NC black bears, respectively. Lastly, the average expected heterozygosity (H_e) for the Western NC black bears was 0.711 (range = 0.413–0.888), while for the Eastern NC black bears was 0.688 (range = 0.543–0.917). Allele frequencies are listed in Supplemental Information.

When examining the results from the ParallelSTRUCTURE analyses, both Evanno's Best K and Pr(X|K) indicated the number of sub-populations among the NC black bears evaluated was most likely three. Among the bears analyzed in this study, the majority of Eastern samples remained in one tight cluster, while the Western samples were split between two clusters and more admixture was observed (Fig. 2). Notably, a single Eastern black bear (out of 57) clustered with black bears collected from Western NC counties, whereas no Western black bear samples clustered with individuals collected from Eastern NC counties. When California black bears were included in the analysis, a separate fourth cluster of CA black bears was recovered along with the original three NC black bear clusters.

The DAPC result for the original populations indicated genetic differentiation between black bears collected from Eastern (n = 57) and Western (n = 116) localities, with little overlap between the sample populations (Fig. 3a). The subsequent cluster analysis indicated that the optimal number of population clusters for all NC black bear samples was four (Fig. 3b). The results from this sub-structuring analysis identified that Eastern black bears predominantly clustered together, and the additional three clusters were comprised of mostly Western individuals (Fig. 3b). The first discriminant axis explained the most variance and separated the cluster containing the Eastern black bear individuals from three clusters of Western black bears. The remaining two discriminant axes highlighted differentiation between the three Western black bear clusters (Fig. 3b). Notably, clusters 3 and 4 consist mostly of individuals from the 3rd cluster in the ParallelSTRUCTURE analysis (dark green; Fig. 2). When examining the individuals within each of the three Western black bears clusters, no trend was observed with respect to county or year of collection.

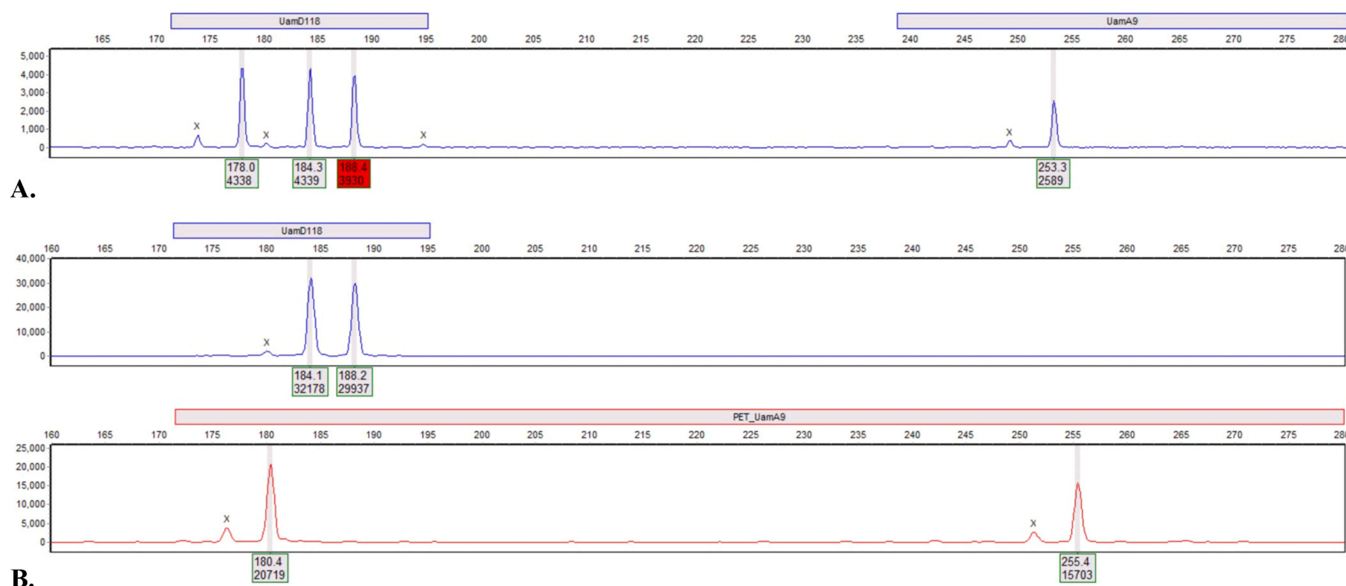

Fig. 4. Example of a genotyping profile of a NC black bear with a novel UamA9 allele. (A) Overlap of a small novel UamA9 allele (shown at 178 bp) into the expected UamD118 marker range when genotyped with the UrsaPlex v2.0 primer multiplex. (B) Confirmation of the novel UamA9 allele (shown at ~180 bp) via a duplex amplification, where a different dye was incorporated into the UamA9 amplicon (PET, red channel), while keeping the same initial dye for UamD118 (6-FAM, blue channel). Electropherogram images are restricted to the two markers of interest. Labels give (top to bottom) peak midpoint (base pair) and peak height (relative fluorescence units; RFUs).

3.3. UamA9 novel alleles

Initial UrsaPlex v2.0 genotyping results showed more than two peaks or unexpected alleles within the UamD118 marker range (176–192 bp) for ~18.6 % of samples (Fig. 4a). Single plex amplification and genotyping of both UamD118 and neighboring UamA9 confirmed that alleles ~180 bp in length were amplifying with the UamA9 primers, a fragment size range ~60 bp lower than reported for California black bears when genotyped with the original UrsaPlex panel [2]. To ensure alleles were correctly assigned for UamA9 in the present study, reamplifications with a duplex of only UamD118 and UamA9 using different dyes (Fig. 4b) were completed for samples in which more than two peaks or unexpected alleles were observed within the UamD118 marker range. Future studies have a number of options to avoid marker overlap, similar to those described previously in [2]: (1) retain all markers with the existing dyes (Table 1) and amplify UamA9 separately to prevent overlap in size ranges, (2) exclude UamD118 from the multiplex, given it offers less discriminatory power than UamA9 (Table 1), and (3) use a genetic analyzer system that can accommodate six different dyes, allowing UamA9 to be moved to a separate dye channel.

4. Conclusions

Overall, the updated UrsaPlex v2.0 panel demonstrated robustness across genomic DNA isolated from multiple biological sample types and sufficient genetic diversity for individual identification of NC black bears. While the samples available for this study contained a high percentage of related individuals, the final dataset of 173 NC black bears displayed PID values low enough for forensic applications, due to the large number of markers included within UrsaPlex v2.0 and their high allele diversity. Genotyping of the NC population revealed novel alleles in the UamA9 locus, which will allow a new version of the panel (currently in development) to accommodate the variation within this informative locus. Further, the utility of UrsaPlex v2.0 for population assignment of NC black bears could be helpful when implementing and monitoring population management strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsiae.2023.100075.

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