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The 'individualization' of large North American mammals

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Summary
The enforcement of wildlife laws and the captive breeding of threatened/endangered species requires the ability to identify individual animals. DNA profiles of a variety of large North American mammals, birds, and fish were generated using ten different oligonucleotide probes. The probes tested were four multilocus probes [33.6, 33.15, JE46, and $(TGTC)_5$] and six 'human unilocus' probes [MS1 (D1S7), CMM101 (D14S13), YNH24 (D2S44), EFD52 (D17S26), TBQ7 (D10S28), and MS43 (D12S11). Each of the probes was chemically synthesized, and labeled by the attachment of alkaline phosphatase; after hybridization, the probes were detected by chemiluminescence catalyzed by the enzyme. Initial screenings against zoo blots including samples of bear, wolf, large cat, wild sheep, deer, birds, marine mammals, and fish indicated that three multilocus probes [33.15, 33.6, $(TGTC)_5$] gave informative patterns containing 15–40 bands for most or all of the animals tested, as did two of the 'human unilocus' probes (MS1 and CMM101). The other five probes appeared informative only in some species (for example, YNH24 against canids). Subsequent screenings of populations within species were used to determine genetic diversity by analysis of observed bandsharing (S). Large heterologous populations, such as white-tailed deer, exhibited highly diverse band patterns ($S \leq 0.2$). Geographically isolated and/or genetically constricted animals, such as endangered Mexican wolves, Tule elk, and Columbian white-tailed deer, exhibited much higher frequencies of bandsharing ($0.6 \leq S \leq 0.95$). Comparison of apparent bandsharing on the same individuals between different probes suggested that the loci detected by 33.15, 33.6, MS1, and CMM101 in animals do not overlap significantly ($0.06 \leq S \leq 0.18$). Databases collected in this manner are being used in national and international forensic casework.

Introduction

Accurate estimates of the magnitude of wildlife crime are difficult. Observations of animal species in the world pet trade include at least 140 mammal species, 85 bird species, and 57 reptile and amphibian species. The global market for illegal wildlife products has escalated into an estimated $1–$2 billion a year business (World Wildlife Fund). Fish & Wildlife Service estimates are $200 million per year in the U.S. alone, a 100% increase in the last ten years. In the US, it is estimated that two animals are poached for every animal taken legally.

The enforcement of laws concerning these crimes is not a simple task. The importation of nearly all wildlife and their parts or products is variously regulated in the US by the Endangered Species Act, Marine Mammal Protection Act, Migratory Bird Treaty Act, Tariff Classification Act, Lacey Act, and the Convention on International Trade of

Endangered Species (CITES). In addition, many species are potential targets of organized poaching interests: black bear (pharmaceuticals), elk (trophy hunting & captive breeding stock), wolf (trophy hunting & predator control) and walrus (ivory). We were interested in determining the extent DNA fingerprint variation could be used to individualize evidence samples from these species.

Individual-specific DNA profiles generated by multilocus (Jeffreys et al., 1985) or unilocus (Nakamura et al., 1987) VNTR probes have been extensively studied in humans. DNA profiles of wildlife have largely been generated with multilocus probes, which crosshybridize (somewhat unpredictably) with many animals (Jeffreys and Morton, 1987; Longmire et al., 1988; Gilbert et al., 1990; Wolfes et al., 1991). In a wildlife forensic setting, techniques to accurately measure genetic diversity are necessary for enforcement of wildlife laws. The ability to identify the smallest units of genetic 'diversity' (i.e., individual animals) also provides basic information for management of endangered or threatened species in captive breeding programs. We were interested in determining the informative value, if any, of both multi- and uni-locus probes in wildlife studies. The work reported here summarizes the preliminary results obtained by screening ten oligonucleotide probes against a number of large mammals (canids, bear, deer, cat, otter, walrus), birds, and fish found in North America. Studies of selected populations were used to establish the level of genetic diversity identified by each probe for a given species.

Forensic casework requires additional considerations, such as assay speed and sensitivity. To address these issues, the work described here was limited to oligonucleotide probes labeled with alkaline phosphatase (Jablonski et al., 1986; Edman et al., 1988; Ruth, 1991), and detected by chemiluminescence (Tizard et al., 1990; Ruth, 1991). Known sensitivities for internally-labeled oligonucleotides are $\approx 2 \times 10^5$ molecules of target sequence (3×10^{-19} mole), or about 50–100 pg of total human DNA for probe 33.6; probes labeled with enzyme on the 5′-end may be less sensitive (Ruth, unpublished results). The application and sensitivities in non-humans were largely unknown.

Materials and methods

DNA was prepared from muscle tissue obtained from hunter-killed animals, or from white blood cells (Jeffreys and Morton, 1987) fractionated from whole blood taken by wildlife biologists from immobilized animals. DNA (5 μg) was digested with 50 units of restriction enzyme (*Pst* I, *Alu* I, *Hae* III, or *Hinf* I) in 20 μl volumes. DNA restriction fragments were electrophoresed through 200 ml 15 × 25 cm long 1.0% agarose gels at 45 volts for 24 hours with Tris-Borate-EDTA buffer.

DNA fragments were transferred to nylon filter membranes (Magnagraph, MSI) by vacuum transfer (LKB VacuGene, Pharmacia) followed by crosslinking with 120 mjoules UV for 30 sec (Stratalinker, Stratagene). Membranes were prehybridized/blocked for 30 minutes at 50°C in hybridization buffer (5 × SSC, pH 7.0, 1% SDS, 0.5% BSA, 0.2% Hammarsten-grade casein, μfiltered). Hybridization was with 2–3 nM alkaline phosphatase-labeled oligonucleotide probe (Edman, 1988; Ruth, 1991; see Tab. 1 for sequences) for 30 minutes at 50°C. Membranes were then washed three times 8–10 min in 1 × SSC, 1% SDS at 50°C, and twice for 5–7 min in 1 × SSC at ambient temperature. Membranes were washed twice in DEA substrate buffer (100 mM diethanolamine, 1 mM $MgCl_2$, pH 10) for 5 minutes at room temperature and then incubated in DEA substrate buffer with 400 μM AMPPD (1,2-dioxetane, Tropix) for 10 minutes. The membranes were exposed to Kodak XAR-5 film for 30 minutes to 3 hours after 1–15 hours incubation in the dark at ambient temperature. For reprobing, the membrane was stripped by washing twice in 0.1 × SSC, 0.5% SDS at 95°C for five minutes and once in 1 × SSC, 1% SDS at room temperature for five minutes.

A semi-automated video imaging system (JAVA, Jandel Scientific) was used to characterize and digitally record each DNA fingerprint profile. All uniquely sized restriction fragments between 2.0–24 kb were scored as to their presence or absence in the individuals being compared. Restriction fragment bands of the same migration (±0.1 mm) and intensity were scored as identical. Band size was determined by comparison to molecular markers (*Hind* III-restricted lambda DNA and 'Genetic Analysis' ladder, Promega). Genetic variation was assessed in each species by making repetitive pairwise comparisons between the DNA fingerprints of individual samples. This was accomplished with "COM*BAND" a Lotus 123™ macro program (Bruce Taylor, USFWS Forensic Lab) which calculated the probability of bandsharing or similarity (S) between two individuals as the number of fragments of equivalent length identified in both DNA fingerprints divided by the total number of fragments compared (Lynch, 1988).

Results and discussion

Oligonucleotide fingerprinting with chemiluminescent detection was used to characterize genetic variability in wildlife species. This approach allows blotting, hybridization, and detection in as little as 8 hours, in contrast to the 4–7 days required to record an isotopic result. This greatly reduces turn-around time in forensic casework.

The ten oligonucleotide probes listed in Tab. 1 were screened against 'zoo' blots of isolated DNAs from representative species of wildlife.

Table 1. The oligonucleotide probes below are labelled with alkaline phosphatase on a thymidine base analog (T) (Jablonski et al., 1986) and used as multilocus DNA typing probes. The probes were synthesized on a custom basis by Syngene, Inc (San Diego, CA)

Common name	Human locus	Length	Sequence
33.15	—	32	5′-AGA GGT GGG CAG GTG GAG AGG TGG GCA GGT GG
33.6	—	22	5′-TGG AGG AGG GCT GGA GGA GGG C
MS1	D1S7	26	5′-AGG GTG GA(CT) AGG GTG GA(CT) AGG GTG GA
CMM101	D14S13	30	5′-TCC ACC TCA GCC CCC TCC ACC TCA GCC CCC
$(TGTC)_5$	—	19	5′-TGT CTG TCT GTC TGT CTG T
YNH24	D2S44	31	5′-AAC AAC CCC ACT GTA CTT CCC ACT GCT CCT G
EFD52	D17S26	27	5′-TAC TAG CAC (AT)(CG)(CT) CCT GG(CT) TAC TAG CAC
TBQ7	D10S28	31	5′-TGC CTG AGC CTT CTC ACA GTC TCA CCT GAT C
MS43	D12S11	25	5′-CCT TCC CGG GGC CCT CCC TAT ACC C
JE46	—	17	5′-CCC CCC GTG TCG CTG TT

Many of the human-derived probes (EFD52, YNH24, TBQ7, MS43, and JE46) hybridized weakly, if at all, with most of the species tested; some reacted with only a few species (example: YNH24 in wolves and birds) under the conditions used. As expected, the multilocus probes 33.6, 33.15, and $(TGTC)_5$ hybridized significantly to most or all species (for example, see Fig. 1). Two human-derived unilocus probes, MS1 and CMM101, also hybridized strongly to all animals tested, resulting in multilocus profiles (for example, see Fig. 2).

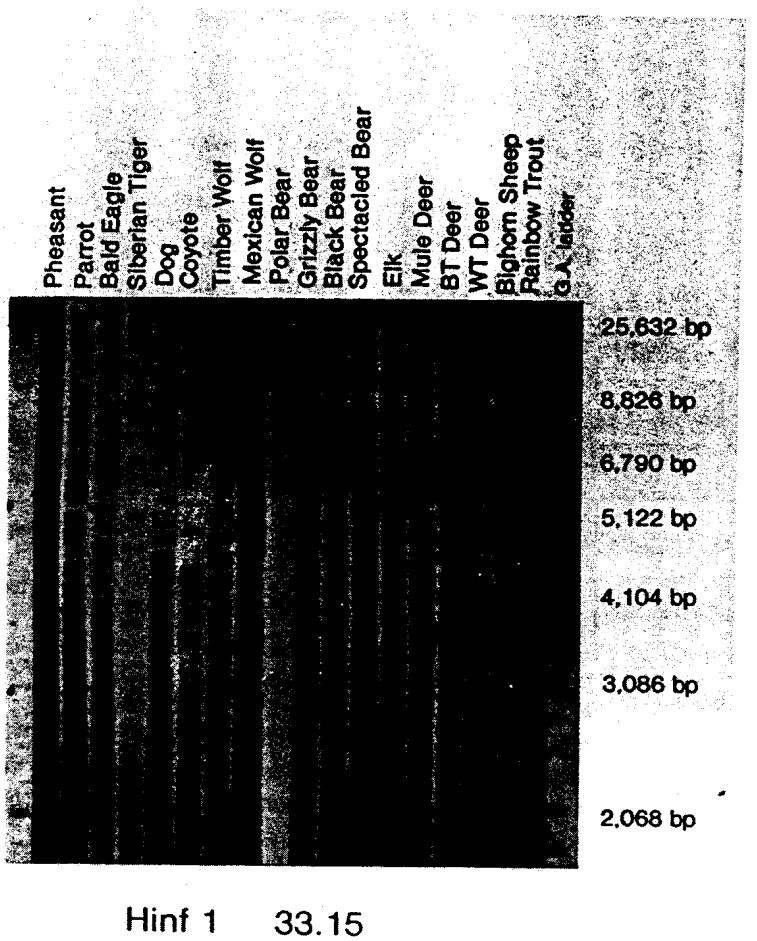

Figure 1. Example of a chemiluminescent 'zoo blot' obtained by restriction of genomic DNA with *Hinf*I and hybridization with an alkaline phosphatase-conjugated oligonucleotide corresponding to the core sequence of the human mini-satellite 33.15. See text for details.

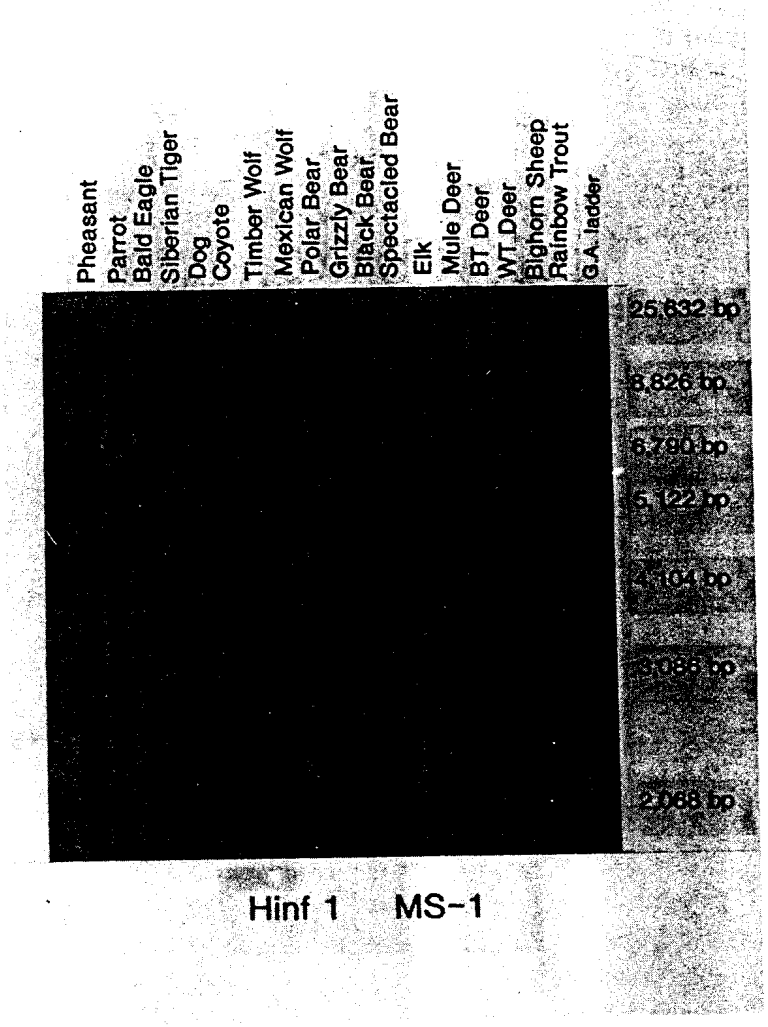

Figure 2. Example of a chemiluminescent 'zoo blot' obtained by restriction of genomic DNA with *Hinf*I and hybridization with an alkaline phosphatase-conjugated oligonucleotide corresponding to the core sequence of the human VNTR MS1. See text for details.

After initial screenings, numerous animals from populations of each species were analyzed, and their DNA profiles compared to establish average similarities (S). The derived similarities for probes 33.15, 33.6, MS1 and CMM101 ranged from $S = 0.2$ to as high as $S = 0.9$, depending on the species tested (see Tab. 2). Correspondingly, the conservative probability (S^{x^2}, where x = average number of bands in a profile) that two unrelated animals would have identical profiles using a *single* probe ranged from 5.5×10^{-8} (1 in ≈ 18 million) for mule deer using 33.6, to only 0.62 (1 in ≈ 2) for elk using MS1.

Table 2. Initial results. Large North American and Marine Mammals

Species	Probe used Ave. Fraction of Bands Shared (Ave. band number per individual) 33.15	33.6	MS-1
Black Bear ($n = 54$)	0.55 ± 0.08 (11)	—	0.79 ± 0.07 (13)
Mexican Wolf ($n = 4$)	0.79 ± 0.06 (21)	0.79 ± 0.06 (21)	0.70 ± 0.07 (12)
Elk ($n = 53$)	0.61 ± 0.08 (20)	0.52 ± 0.08 (15)	0.91 ± 0.12 (9)
Mule Deer ($n = 36$)	—	0.29 ± 0.01 (27)	—
WT Deer ($n = 72$)	—	0.20 ± 0.01 (15)	—
Col WT Deer ($n = 18$)	0.54 ± 0.01 (16)	0.61 ± 0.01 (24)	0.62 ± 0.02 (27)
Walrus ($n = 58$)	0.43 ± 0.08 (19)	0.40 ± 0.08 (22)	—
Sea Otter ($n = 18$)	0.60 ± 0.08 (14)	—	—

Black Bear –	*Ursus americanus* (CITES Appendix II)
Mexican Wolf –	*Canis lupus baileyi* (CITES Appendix II)
North American Elk –	*Cervus elaphus*
Mule Deer –	*Odocoileus hemionus hemionus*
White-tailed (WT) Deer –	*Odocoileus virginianus*
Columbian WT Deer –	*Odocileus virginianus leucurus* (endangered)
Pacific Walrus –	*Odobenus rosmarus divergens* (Marine Mammal Protection Act)
Sea Otter –	*Enhydra lutris* (Marine Mammal Protection Act)

These exclusion powers can be increased in a multiplicative manner by the use of probe combinations, but only if apparent bands ('loci') between probes are not shared. As a measure of overlap, apparent "bandsharing" *between* different probes on the same individuals was determined. In the endangered Columbian white-tailed deer ($S \approx 0.6$), the results *between* all possible probe combinations of 33.15, 33.6, MS1 and CMM101 ranged from $S = 0.06$ to $S = 0.18$; this indicates that any apparently common bands which may exist are indistinguishable from background band sharing. As a result, even species with little genetic variation can be identified with exclusion probabilities of 1 in more than 10^6 individuals, representing adequate discrimination for wildlife forensics.

We have used such techniques and analyses in more than 150 forensic cases in North America. The analyses have involved such species as elk, deer, wolf, parrots, bighorn sheep, eagle, and bear. Such cases generally involve identification of individuals, matching field evidence (blood, tissue, gut pile, etc.) to evidence (such as meat or trophies) in possession of the suspect. Occasionally, parentage determinations are required, as well. It is our hope that the wildlife DNA fingerprinting techniques and results described in this paper will simplify the enforcement of wildlife laws and aid in the conservation of wildlife for the future.

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References

Edman JC, Evans-Holm ME, Marich JE, Ruth JL (1988) Rapid DNA fingerprinting using alkaline phosphatase-conjugated oligonucleotides. Nucleic Acids Res 16: 6235

Gilbert DA, Lehman N, O'Brien SJ, Wayne RK (1990) Genetic fingerprinting reflects population in the California Channel Island fox. Nature 344: 764–767

Jablonski E, Moomaw E, Tullis R, Ruth JL (1986) Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. Nucleic Acids Res 14: 6115–6128

Jeffreys AJ, Wilson V, Thein SL (1985a) Hypervariable 'minisatellite' regions in human DNA. Nature 314: 67–73; Jeffreys AJ, Wilson V, Thein SL (1985b) Individual-specific 'fingerprints' of human DNA. Nature 316: 76–79

Jeffreys AJ, Morton DB (1987) DNA fingerprints of dogs and cats. Animal Genetics 18: 1–15

Longmire JL, Lewis AK, Brown NC, Buckingham JM, Clark LM, Jones MD, Meincke LJ, Meyne J, Ratliff RL, Ray FA, Wagner RP, Moyzis RK (1988) Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family *Falconidae*. Genomics 2: 14–24

Lynch M (1988) Estimation of relatedness by DNA fingerprinting. Mol Biol Evol 5: 584–599

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E, White R (1987) Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235: 1616–1622

Ruth JL (1991) Oligodeoxynucleotides with reporter groups attached to the base. *In*: Eckstein F (ed.), Oligonucleotides and analogues: A practical approach, Oxford University Press, pp 255–282

Tizard R, Cate RL, Ramachandran KL, Wysk M, Voyta JC, Murphy OJ, Bronstein I (1990) Imaging of DNA sequences with chemiluminescence. Proc Natl Acad Sci USA 87: 4514–4518

Wolfes R, Mathe J, Seitz A (1991) Forensics of birds of prey by DNA fingerprinting with ^{32}P-labeled oligonucleotide probes. Electrophoresis 12: 175–180