非放射性オリゴヌクレオチドプローブを用いたキタオットセイのDNAフィンガープリンティング

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Non-radioactive Oligonucleotide Probes for DNA Fingerprinting in Northern Fur Seals

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Non-isotopic synthetic oligonucleotides labeled with horseradish peroxidase were used as DNA fingerprinting probes for northern fur seals, *Callorhinus ursinus*. Of the eight probes screened, three oligonucleotides composed of simple repetitive sequences, $(GGAT)_4$, $(TCC)_5$ and $(CA)_8$, could generate DNA fingerprints. The resultant DNA fingerprints were analyzed, and parameters were calculated, such as average number of bands and band-sharing rate between unrelated individuals and mother-offspring pairs. The most informative DNA fingerprints were obtained by $(GGAT)_4$ hybridized to *Hin*f I-digested DNA. Average likelihood of two unrelated individuals to share an identical fingerprint was as low as 1.46×10^{-6}, and the probability of missassignment in paternity assignment was 9.23×10^{-6}. The method provides a fast non-hazardous tool for individualization and paternity testing in northern fur seals.

Key words: DNA fingerprinting, oligonucleotides, simple repetitive sequence, northern fur seal

Introduction

A number of molecular methods have been developed for analysis of genetic variability in human and wildlife populations. DNA fingerprinting is one of the efficient methods which detects differences in number of highly repetitive sequences at multiple loci as restriction fragment length polymorphisms (RFLPs). The method was first discovered by Jeffreys et al. (1985a) during analysis of human myoglobine gene, and then applied for individualization purpose (Jeffreys et al., 1985b). Now it is widely used in forensic medicine and in wildlife biology for individualization, parentage assessment and measurement of genetic relatedness.

Otariid seals have polygynous mating systems often called incorrectly as harems, but the actual social structure of breeding colonies should be reevaluated based on a quantitative measurement of reproductive success and relatedness between individuals (Boness, 1991). DNA fingerprinting is expected as a powerful tool to analyze the mating systems of pinnipeds (Boness et al., 1993). It is also effective in pedigree control of captive seals because casual observations of copulation are not always a reliable index of paternity (Harris et al., 1991).

The original method of DNA fingerprinting invented by Jeffreys et al. (1985a, b) uses radioactively labeled recombinant probes. Laborious cloning and hazardous use of radioisotopes have imposed an obstacle to the wide application of DNA fingerprinting. Recently, chemically synthesized oligonucleotides are used for DNA ingerprinting probes in various species including domestic and wild animals (Epplen et al., 1991). In some of the published methods, oligonucleotide probes are coupled with non-radioactive detection systems based on chemiluminescence, bioluminescence or colorimetric reaction catalyzed by marker enzymes (Zischler et al., 1989, 1991; Nagamori et al., 1990; Pena et al., 1991). If non-radioacitve oligonucleotides are efficient enough for DNA fingerprinting in pinnipeds, it will provide a fast and non-hazardous tool for practical analysis of individualization, paternity and relatedness.

In this study, we tested the use of non-radioactive oligonucleotides for DNA fingerprinting in northern fur seals, *Callorhinus ursinus*. A panel of oligonucleotides were hybridized to restriction enzyme digested DNA of northern fur seals and detected by the chemiluminescence systems. Band number and band-sharing rates of the resulting DNA fingerprints were analyzed to estimate the accuracy in individualization and paternity assessment.

Materials and Methods

DNA samples

Tissue samples were collected from six female northern fur seals and their fetuses taken off the Pacific coast of northeastern Japan in April, 1994. Tips of hind flippers were cut off and preserved frozen at -20℃ until use. Tissue samples were also biopsied from hind flippers of one adult female, her pup and an adult male (putative father of the pup) on St. Paul Island, Alaska in 1993 and 1994. The latter samples were preserved in 20% DMSO/saturated NaCl solution for 2-12 months (Seutin et al., 1991) and later kept frozen at -80℃.

DNA extraction and restriction

Tissue samples (0.1-0.5 g) were minced and digested in 5 ml of Proteinase K lysis buffer (10

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Table 1. List of oligonucleotide probes used for DNA fingerprinting in this study.

Probe	Sequence	Base number	Tm*
$(GGAT)_4$	5'- GGA TGG ATG GAT GGA T -3'	16mer	48℃
$(GACA)_4$	5'- GAC AGA CAG ACA GAC A -3'	16mer	48℃
$(GATA)_4$	5'- GAT AGA TAG ATA GAT A -3'	16mer	40℃
$(CAC)_5$	5'- CAC CAC CAC CAC CAC -3'	15mer	50℃
$(TCC)_5$	5'- TCC TCC TCC TCC TCC -3'	15mer	50℃
$(CA)_8$	5'- CAC ACA CAC ACA CAC A -3'	16mer	48℃
myo-33	5'- GGA GGT GGG CAG GAA GGA CCG AGG TGT AAA GCT -3'	33mer	106℃
33.15 core	5'- GAG GTG GGC AGG TGG A -3'	16mer	54℃

*: Calculated from the formula ; Tm=4×(number of G and C) + 2×(number of A and T)

mM Tris-HCl, 10 mM EDTA, pH. 8.0, 0.1 mg/ml Proteinase K, 0.3% SDS) overnight at 57℃. The lysate was extracted twice with phenol/chloroform/isoamilalcohol and once with chloroform/isoamilalcohol. DNA was precipitated by adding 1/10 volume of 3 M CH_3COONa and an equal volume of isopropanol. DNA was pelleted by centrifuging for 15 min at 3500 rpm, washed with 75 % ethanol, dried, and resuspended in low TE buffer. RNA was removed with 20 μg/ml RNase for 2 h at 37℃. The solution was incubated with 0.1 mg/ml Proteinase K for 5 h, extracted with phenol and chloroform, and ethanol precipitated. The resultant DNA pellet was centrifuged (12000 rpm, 15 min), washed, dried, and resuspended in low-TE buffer.

DNA samples were cleaved with 3 U/μg DNA of the restriction endonucleases *Hae* III or *Hin*f I as recommended by the manufacturer. DNA was purified by phenol chloroform extraction and ethanol precipitated.

Electrophoresis and Southern transfer

Digested DNA (3-5 μg) was electrophoresed on 10×8 cm 1% agarose gels or 20×15 cm 0.8% agarose gels in TBE buffer at 1 V/cm for up to 48 h until fragments smaller than 1kb ran off the gel. After electrophoresis, DNA was denatured in 0.2 M NaOH, 0.6 M NaCl for 30 min followed by neutralization in 0.24 M Tris-HCl/0.6 M NaCl (pH 7.5). The DNA was transferred onto nylon membrane by vacuum or capillary transfer and fixed to it by baking for 2 h at 80℃.

Preparation of oligonucleotide probes

Two types of oligonucleotides were used as DNA probes in this study. The first ones consisted of simple repeats of two to four sequences specific for microsatellite DNA, i.e., $(GGAT)_4$, $(GACA)_4$, $(TCC)_5$, $(CAC)_5$, $(CA)_8$ and $(GATA)_4$. The second ones were repeat units of minisatellite probes, i.e., core sequences of Jeffreys et al.'s (1985) 33.15 and myo probes (Table 1). These oligonucleotides were synthesized in an Applied Biosystems Synthesizer and purified by a commercial company (Japan Biosystems Inc., Tokyo).

Hybridization

ECL® -3' oligolabeling systems (Amersham International plc, Buckinghamshire, England) were used for labeling oligonucleotide. The ECL® system detects target nucleotides by chemiluminescence reaction catalyzed by horseradish peroxidase (HRP) combined to the DNA probe (Durrant, 1995). Labeling of oligonucleotide and hybridization, blocking, antibody treatment, and signal detection were undertaken according to the manufacturer's standard protocol. After hybridization for 6 h to overnight, the blot was washed twice with 5×SSC for 5min at RT followed by one stringency wash with 5×SSC, 0.1% SDS for 15 min. Hybridization and stringency wash was performed at Tm-10℃ except myo-33 which was hybridized at 50℃. Then the blot was blocked, treated with HRP-antibody conjugate, and detected onto blue light sensitive X-ray film. The blot was boiled in 0.1% SDS for 3 min to remove hybridized probe before reprobing with other probes.

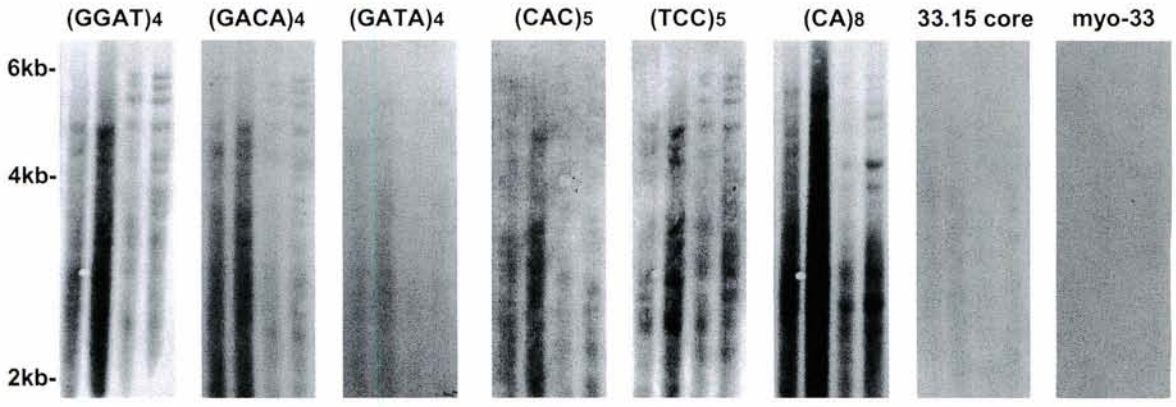

Fig. 1. Screening of eight different oligonucleotides for generating fingerprints with *Hae* III digested (left two lanes) or *Hinf* I digested (right two lanes) DNA of two northern fur seals.

Table 2. Results of Southern hybridization of six different oligouncleotides to *Hae* III-digested DNA and *Hinf*-I digested DNA extracted from two northern fur seals.

Probe	Hybridization temperature	*Hae* III digest			*Hinf* I digest		
		Band no.	Size range	Smear	Band no.	Size range	Smear
$(GGAT)_4$	38℃	2−3	4−5 kb	+++	8−9	3.5−6	+
$(GACA)_4$	38℃	3	4−5	++	8−9	3−6	+
$(TCC)_5$	40℃	3	4−4.5	+	4	4.5−6	+
$(CAC)_5$	40℃	0	−	+	0	−	+
$(CA)_8$	37℃	1	6	+++	7	3.5−6	+++
$(GATA)_4$	30℃	0	−	+	0	−	+
33.15 core	44℃	0	−	+	0	−	+
myo-33	50℃	0	−	−	0	−	−

Evaluation of DNA fingerprints

We determined the number of bands per individual for each DNA fingerprints. The band-sharing rates between two individuals were calculated following the equation of Cummings and Hallett (1991):

$$D = 2N_{ab} / (N_a + N_b)$$

where N_{ab} is the number of bands common to individuals a and b, and N_a and N_b are the total number of bands obtained for individuals a and b, respectively. Differences in the average probability of band-sharing ($\bar{D}$) between six pairs of mother-offspring and between five pairs of unrelated females and pups were examined. Average probability of errors in individual identification and paternity assignment was calculated using the band-sharing rate. The average likelihood of two unrelated individuals to share all their bands is

$$\bar{P} = \bar{D}_{nr}^{\bar{N}}$$

where $\bar{D}_{nr}$ is average band-sharing rate between non-related individual, and $\bar{N}$ is average number of bands per individual. Probability of misassignment in paternity test was

$$F = D_{nr}^{(\bar{N}-\bar{N}mp)} = D_{nr}^{N(1-\bar{D}mp)}$$

where $\bar{N}_{mp}$ is average number of bands common to a mother and her offspring and $\bar{D}_{mp}$ is average probability of band-sharing between a mother and her offspring.

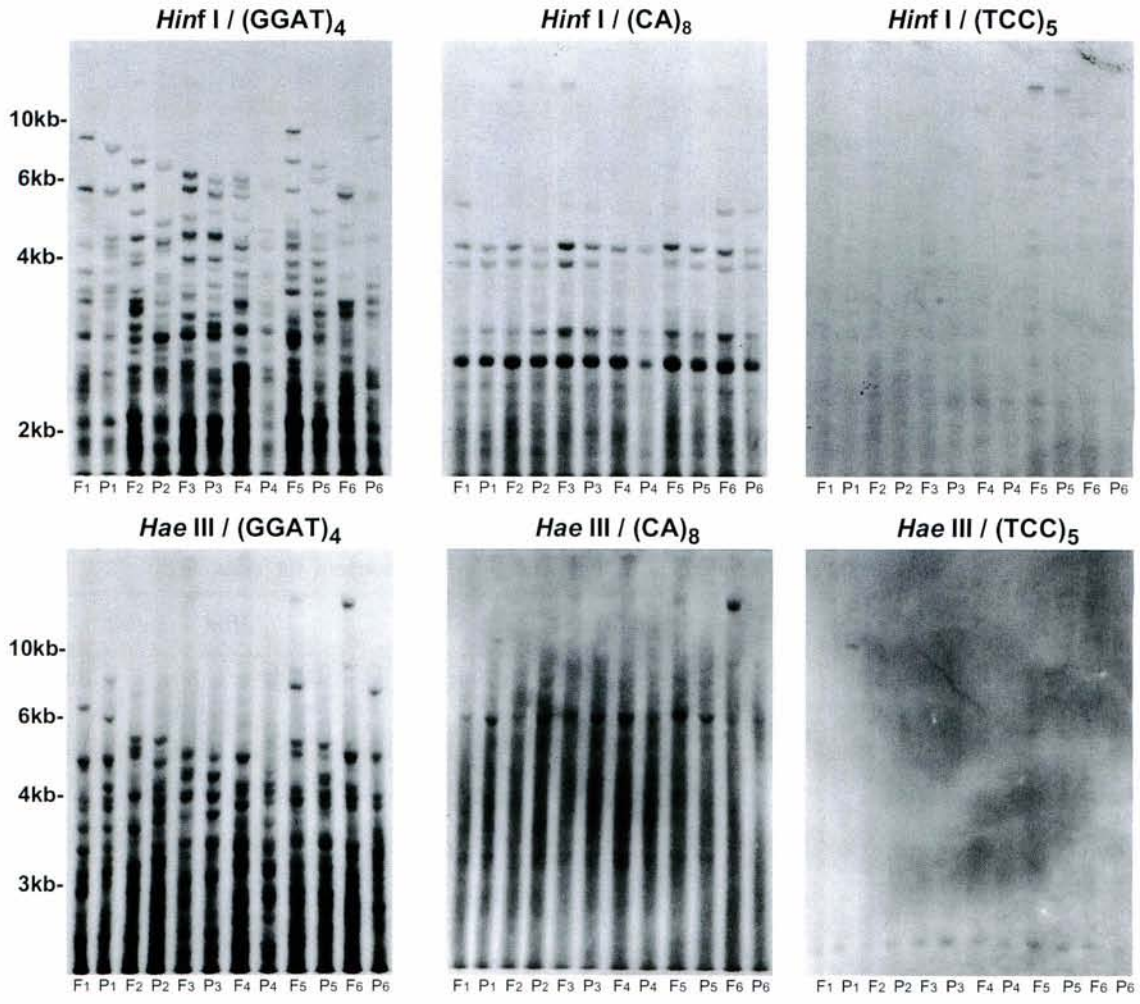

Fig. 2. Fingerprints of northern fur seals obtained using three oligonulceotide probes hybridized to genomic DNA from six mothers (F1-F6) and their pups (P1-P6) digested by endonuclease *Hae* III or *Hinf* I.

Results

Screening of oligonucleotide probes

In the first set of experiments, DNA from two northern fur seals was cleaved by *Hae* III or *Hinf* I and hybridized to different oligonucleotide probes to examine fingerprint patterns (Fig. 1). Hybridization to $(GGAT)_4$, $(TCC)_5$ and $(CA)_8$ revealed several bands of fragments between 3-6 kb either with *Hae* III digested DNA or with *Hinf* I digests. Bands were fewer and weaker in *Hae* III digested DNA masked by stronger signals of background smear compared to *Hinf* I digests. $(GACA)_4$ generated a similar number of weak bands for both *Hae* III and *Hinf* I digested DNA. On the contrary, no bands were obtained by hybridization to $(CAC)_5$, $(GATA)_4$, 33.15 core and myo-33 (Table 2).

Evaluation of fingerprints

We next compared band-sharing rates between mother-offspring and non-relatives to evaluate effectiveness of three probes, $(GGAT)_4$, $(TCC)_5$ and $(CA)_8$, in individualization and relatedness analysis. *Hinf* I and *Hae* III digested DNA extracted from six pairs of mother-pup and five pair of female and non-related pup were electrophoresed over adjacent lanes of an agarose gel (Fig. 2). Fragments larger than 2 kb were counted because smaller fragments were masked by the strong background. Average number of bands and average rate of band-sharing are compared in Table 3.

Clear polymorphic bands were obtained when $(GGAT)_4$ probe was hybridized to *Hinf* I digested DNA. $(CA)_8$ detected clear bands but most of them were not polymorphic. (TCC_5) generated small number of polymorphic bands (Table 3).

With the figures in Table 3, the effectiveness of each probe/enzyme combination in individualization and paternity assignment was calculated. Combination of $(GGAT)_4$ probe and restriction enzyme *Hinf* I showed the lowest probability of error, 1.46×10^{-6} for individual identification and 9.23×10^{-4} for paternity tests (Table 4).

Test application

A test application of DNA fingerprinting was done for wild northern fur seals using the above described methods. $(GGAT)_4$ was hybridized to genomic DNA of a female, her pup cleaved with restriction endonucleases *Hinf* I or *Hae* III (Fig. 3). *Hinf* I digested DNA of the pup generated 16 bands, of which nine were common to its mother. All the

Table 3. Comparison of band-sharing rates between mother-offspring and between non-relatives.

Probe	Enzyme	Average no. of bands	Band-sharing rate	
			Mother-offspring	Unrelated
$(GGAT)_4$	*Hinf* I	12.8±2.60	0.48±0.09*	0.35±0.11*
$(GGAT)_4$	*Hae* III	8.5±1.62	0.66±0.17*	0.34±0.11*
$(CA)_8$	*Hinf* I	7.4±1.83	0.83±0.11	0.74±0.15
$(CA)_8$	*Hae* III	4.1±1.83	0.72±0.20	0.53±0.27
$(TCC)_5$	*Hinf* I	4.1±2.15	0.58±0.21*	0.29±0.10*

*Difference significant at $p<0.05$ (t −test)

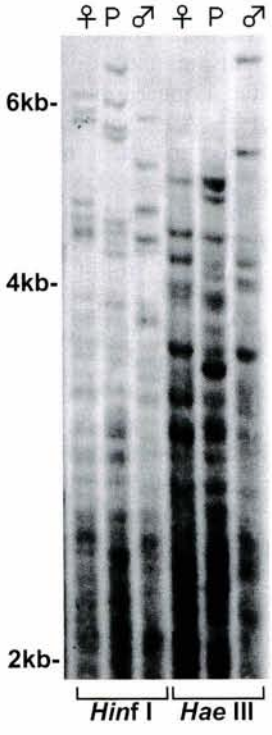

Fig. 3. Test application of DNA fingerprinting for paternity assessment in northern fur seals. DNA from a female, her pup and an adult male was digested by *Hae* III or *Hinf* I and southern hybridized to $(GGAT)_4$ labeled with horse radished peroxidase.

remaining bands were shared by the putative father. Band-sharing rate of the pup and the putative father was 0.45, higher than the average band-sharing rate in this study. Probability of an unrelated male to share these bands (F) was smaller than 1/1500. Fingerprints from *Hae* III digested DNA showed three paternal bands among nine pup bands, which corresponded to F=0.04. These results strongly suggest that the putative father sired the pup.

Discussion

Oligonucleotides complimentary to simple repeated sequences could generate informative DNA fingerprints in northern fur seals. These oligonucleotide probes have several advantages over the cloned minisatellite probes. Oligonucleotide is synthesized chemically and thus large quantity of probe is readily obtained with a constant quality. Simple tandem repeat probes are applicable to a wide variety of eukaryote genome. For example, $(GGAT)_4$, the most informative probe for northern fur seals in this study, has been used for DNA fingerprinting of plants, fish, birds and mammals (Epplen et al., 1991). Stable quality of synthetic oligonucleotides is competent for routine application of DNA fingerprinting in human clinical diagnostics (Neitzel et al., 1991).

DNA fingerprinting can be used as an effective tool in several fields of wildlife biology and captive husbandry. It has been used to assess paternity and mating success in various species (Burke, 1989; Hoelzel et al., 1991; Amos et al., 1993). However, tandem repeats are known to have a higher mutation rate compared with conventional loci (Schäfer et al., 1988), which may impose errors in parent-offspring analysis. Although we did not check it, assessment of mutation rate of fingerprinting bands using known parent-offspring pairs in captivity will improve the accuracy of paternity assessment. DNA fingerprints are also useful for analysis of relatedness and genetic diversity within and between populations (*e.g.*, Schenk and Kovacs, 1996; Meisfjord and Sundt, 1996; Schaeff et al., 1997).

The non-radioactive oligonucleotide probes

Table 4. Average probability of error in individual identification and paternity test using combinations of oligonucleotide probes and restriction endonucleases for northern fur seal DNA.

Probe	Enzyme	Probability of error	
		Individual identification	Paternity test
$(GGAT)_4$	*Hinf* I	1.46×10^{-6}	9.23×10^{-4}
$(GGAT)_4$	*Hae* III	8.08×10^{-5}	0.04
$(CA)_8$	*Hinf* I	1.08×10^{-1}	0.68
$(CA)_8$	*Hae* III	7.41×10^{-2}	0.48
$(TCC)_5$	*Hinf* I	6.25×10^{-3}	0.12

described above provide a fast and non-hazardous method of DNA fingerprinting without using special facility required for cloning and handling radioactive compounds. It will extend the potential utility of multilocus DNA fingerprints in genetic analysis of wild and captive animals.

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非放射性オリゴヌクレオチドプローブを用いたキタオットセイのDNAフィンガープリンティング

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摘　要

非放射性標識を施した化学合成オリゴヌクレオチドをプローブとして，キタオットセイのDNAフィンガープリントを作成する方法を検討し，得られたDNAフィンガープリントについてバンド数や母子間・非血縁個体間のバンド共有度を求め，個体識別や父子鑑定における有効性を調べた．プローブとして，２～４塩基の短い反復配列よりなる，$(CA)_8$，$(GGAT)_4$，$(GATA)_4$，$(GACA)_4$，$(CAC)_5$，$(TCC)_5$の６種類，およびJeffreys *et al.*, (1985a)の*myo*プローブと33.15プローブの反復単位を用いた．プローブはHRP（西洋ワサビのペルオキシダーゼ）を用いて標識し，HRPが触媒する化学発光により標的DNAを検出した．２種類の制限酵素*Hae* IIIと*Hinf* Iで消化したゲノムDNAと各種プローブをハイブリダイズした結果，$(GGAT)_4$，$(CA)_8$，$(TCC)_5$でフィンガープリントが得られた．いずれの場合も*Hae* IIIよりも*Hinf* Iで消化したDNAの方が多数のバンドが検出された．３種類のプローブを用いたDNAフィンガープリントの，平均バンド数と平均バンド共有度を比較したところ，$(GGAT)_4$と*Hinf* I の組み合わせがバンド数が多く多型性も高かった．$(TCC)_5$はバンド数が少なく，$(CA)_8$は多型性が低かった．$(GGAT)_4$と*Hinf* Iの組み合わせでは，個体識別と父子鑑定の危険率が，それぞれ1.46×10^{-6}，9.23×10^{-4}と低かった．本法は，放射性同位元素を用いない簡便なDNAフィンガープリント法として，キタオットセイの個体識別・父子鑑定に利用可能である．

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