Mar. Biotechnol. 6, 430–434, 2005
DOI: 10.1007/s10126-004-1100-2

MARINE BIOTECHNOLOGY

DNA Microarray for Rapid Detection of Mitochondrial DNA Haplotypes of Chum Salmon

Shogo Moriya,[1] Shigehiko Urawa,[2] Osamu Suzuki,[1] Akihisa Urano,[3] and Syuiti Abe[4,5]

[1]*Research and Development Center, Nisshinbo Industries, Inc., 1-2-3 Onodai, Midori-ku, Chiba 267-0056, Japan*
[2]*Genetics Section, National Salmon Resources Center, 2-2 Nakanoshima, Toyohira-ku, Sapporo 062-0922, Japan*
[3]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan*
[4]*Laboratory of Animal Cytogenetics, Center for Advanced Science and Technology, Hokkaido University, North 10, West 8, Sapporo, Kita-ku 060-0810, Japan*
[5]*Laboratory of Breeding Science, Division of Marine Biosciences, Graduate School of Fisheries Sciences, Hokkaido University, Minato, Hakodate 041-8611, Japan*

Abstract: For use in genetic stock identification, we developed an oligonucleotide (DNA) microarray hybridization method for rapid and accurate detection of nucleotide sequence variations in 20 previously identified variable nucleotide sites in about 500 bp within the 5′ half of the control region of mitochondrial DNA of chum salmon (*Oncorhynchus keta*). The method includes immobilization of synthesized oligonucleotides containing respective polymorphic sites on a glass slide precoated with polycarbodiimide resin, a 2-hour hybridization with DNA microarray of biotinylated polymerase chain reaction fragments spanning the 5′ variable portion followed by short washing, and visualization of hybridization signals by conventional ABC method and scanner-assisted computation of signal intensity on a computer. The entire process of hybridization and detection was completed within 4 hours. The resulting DNA microarray could detect all of the single nucleotide mutations and therefore could be used to identity the sequence variations defining 30 mtDNA haplotypes of chum salmon as revealed previously by nucleotide sequence analysis.

Key words: DNA microarray, chum salmon, mtDNA, haplotype, stock identification.

INTRODUCTION

Recently developed molecular techniques are expected to provide a powerful means to reveal genetic variation in salmon populations with increased accuracy and resolution (Ferguson et al., 1995). Analysis of mitochondrial DNA has received considerable attention in genetic stock identification (GSI) of Pacific salmon species because of the higher sequence variability than single copy nuclear genes (Brown et al., 1979) and clonal haploid inheritance. We recently detected greater variation in the mtDNA control region of chum salmon (*Oncorhynchus keta*) by nucleotide sequence analysis than had been observed by previous analyses of restriction fragment length polymorphisms (RFLPs) (Sato et al., 2001, 2004). Base substitutions and indels observed in 20 sites of the 5′ half of the mtDNA control region defined a total of 30 haplotypes in more than 2000 individuals from

Received April 18, 2003; accepted November 27, 2003; online publication November 18, 2004.
Corresponding author: Shogo Moriya; e-mail: moriya@nisshinbo.co.jp

48 populations collected from Japan, Korea, Russia, and North America, serving as a useful tool for phylogeographic analysis of Pacific Rim populations (Sato et al., 2004). These findings suggest an increased potential for mtDNA sequence analysis to provide better estimation of stock composition in mixed ocean populations of chum salmon (Abe et al., 2002). However, nucleotide sequence analysis requires specialized, expensive laboratory equipment and expert skill. In addition, time-consuming sequence analysis may not be suitable for salmon stock identification that will require a large number of samples.

In place of conventional sequence analysis using slab gel electrophoresis or capillary electrophoresis, hybridization analysis by oligonucleotide (DNA) microarray was suggested to be a promising new technology (Lander, 1999). DNA microarrays typically consist of thousands of DNA or complementary DNA sequences immobilized on a precoated glass slide or substrate, potentially allowing rapid and cost-effective screening of many gene expression profiles and all possible mutations and sequence variations in genomic DNA at once by hybridization of labeled probes (Schena et al., 1995; Hacia, 1999; Lander, 1999). Theoretically, nucleotide sequences can be determined by hybridization of labeled fragments spanning a target region with arrayed oligonucleotides, each having a nucleotide sequence complementary or homologous to a polymorphic site, immobilized on an appropriate substrate.

In the present study we attempted to develop a rapid and accurate method to detect mtDNA haplotypes of chum salmon for GSI using a DNA microarray on a glass slide, which immobilized oligonucleotides containing the reported sequence variations in the control region (Sato et al., 2001, 2004). The obtained DNA microarray detected a single nucleotide mutation; it was able to identify all 30 haplotype sequences that had been revealed in the previous nucleotide sequence analysis (Sato et al., 2001, 2004). The present DNA microarray method will become a practical means for chum salmon GSI in the field or onboard ships at sea.

Materials and Methods

DNA Extraction

Genomic DNA extraction was performed as described previously (Sato et al., 2001), In brief, about 50 μl of liver homogenate from each of 37 individuals, including 34 known and 3 unknown for mtDNA haplotypes, was added to 500 μl sodium Tris EDTA buffer (0.1 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) containing 500 μg/ml proteinase K and 0.5% sodium dodecylsulfate (SDS), and incubated at 37°C overnight. DNA was extracted with a mixture of phenol (250 μl) and 24:1 chloroform–isoamyl alcohol (250 μl) 3 times and then 2 times with 500 μl of 24:1 chloroform–isoamyl alcohol alone. DNA in aqueous phase was recovered by ethanol precipitation, dried in air, and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Polymerase Chain Reaction Amplification

PCR primers used included those for amplification of about 500 bp in the 5′ variable portion of the mtDNA control region (see Sato et al., 2001) and of two fragments spanning that region, which included all 20 variable sites identified previously (Sato et al., 2001, 2004). The sequences of primer pairs for amplification of the two fragments were 5′-AAC TAC TCT CTG GCG GCT-3′ (forward) and 5′-TTG GTG GGT AAA GAC GGA-3′ (reverse); 5′-AGT CCT GCT TAA TGT AGT-3′ (forward) and 5′-ATA AGA TTG ACA CCA TTA-3′ (reverse). These two fragments were amplified separately in a 50 μl reaction mixture containing 25 to 100 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 1 U *Taq* DNA polymerase (TaKaRa), 1 μM each of forward and reverse primers. The latter was biotinylated for simultaneous labeling with PCR amplification for both the single fragment and two fragments. Conditions for the PCR using a DNA engine (MJ Research) were as follows; preheating at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 45°C for 30 seconds, and extension at 72°C for 30 seconds, with postcycling extensions at 72°C for 3 minutes. The PCR products were verified for fragment size by 2% agarose gel electrophoresis and ethidium bromide staining.

Preparation of DNA Microarray

Oligonucleotides for microarray were synthesized at Nisshinbo Industries, Tokyo. The size of oligonucleotides was 17 to 20 mer, and each oligomer contained the previously identified sing nucleotide mutation such as a base substitution or indel (Sato et al., 2001, 2004) in the center of the sequence (Table 1). These oligomers were referred to as ''capture'' oligonucleotides.

Table 1. Oligomers Immobilized to Glass Slide[a]

Variable site	Number	Sequence	Size (mer)	Variable site	Number	Sequence	Size (mer)
10	1	···ACA**T**CCC···	20	194	20	···AGC**A**AAA···	20
	2	···ACA**C**CCC···	20		21	···AGC**T**AAA···	20
30	3	···TGC**T**ATA···	20	231	22	···AAC**T**GAT···	20
	4	···TGC**C**ATA···	20		23	···AAC**C**GAT···	20
42	5	···TAA**A**CCC···	19	242	24	···TGC**C**GCA···	18
	6	···TAA**G**CCC···	19		25	···TGC**T**GCA···	18
57	7	···ACT**A**CAC···	20	250	26	···CAA**T**AAA···	17
	8	···ACT**T**CAC···	20		27	···CAA**C**AAA···	17
70	9	···TAA**T**ATT···	20	260	28	···CCAA**C**TA···	20
	10	···TAA**C**ATT···	20		29	···CCA**G**CTA···	20
79	11	···ATA**T**TAT···	20	339	30	···ATA**TC**AG···	20
	12	···ATA**C**TAT···	20	&340	31	···ATAA**C**AG···	20
96	13	···ATA-TAT···	19		32	···ATAA**T**AG···	20
	14	···ATAA**T**AT···	20	386	33	···AG**G**TC-CT**C**GTG···	20
108	15	···TGC**T**CGT···	20	&395	34	···AG-TC···CTA**G**TG···	19
	16	···TGC**A**CGT···	20	401	35	···GAA**T**TAT···	20
	17	···TGC**C**CGT···	20		36	···GAA**C**TAT···	20
154	18	···TAA**C**CCC···	20	471	37	···TAAAGCA···	20
	19	···TAA**G**CCC···	20		38	···TAA**C**GCA···	20

[a]Variable nucleotides are shown in boldface. Number at variable site shows nucleotide positions from the 5′ end of the mtDNA control region of chum salmon. Immobilized oligomers are coded from 1 to 38. For each oligomer sequence, (see Sato et al. 2001, 2004).

Other oligomers termed ''reference'' oligonucleotides, of the same size as capture oligonucleotides, contained polymorphic sites but had nucleotide sequences complementary or homologous to the sequences that did not show polymorphism. Nucleotide sequences of 30 haplotypes exhibiting the 20 mutation sites have been registered in the DDBJ/EMBL/GenBank databases with accession numbers AB039890 to AB039901 and AB091514 to AB091531.

Immobilization of DNA oligomers to CarboStation glass slides for DNA microarray, originally developed by Nisshinbo Industries, was according to the manufacturer's instruction. Both capture and reference oligomers can be immobilized covalently on this glass slide because its surface is uniformly coated with polycarbodiimide resin; this treatment does not require expensive linkers at either end of an oligomer for binding, so that microarrays can be prepared using oligomers of desired size and sequence. For each oligomer 10 μl dissolved in a Microspotting Solution (TeleChem International Inc.) was spotted on a CarboStation with a spotting machine. Both capture and reference oligomers were spotted in duplicate on the glass slide to obtain stable results. After spotting the glass slide was steamed for several seconds with hot water, irradiated with 600 mJ ultraviolet light by UV Stratalinker (Stratagene), again steamed, and dried on a hot plate. Each oligomer spot in a microarray was coded as shown in Table 1.

The glass slide was immersed in a blocking buffer containing 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Triton-X, and 3% bovine serum albumin at room temperature for 30 minutes. After drying at room temperature, the glass slide was washed in TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA), dried, and refrigerated in dark until use.

Hybridization and Signal Detection

For two PCR fragments, 2 μl each of reaction mixture was mixed and denatured at 100°C for 1 minute, followed by quenching on ice for 5 minutes. For a single PCR fragment, 4 μl of reaction mixture was used. The denatured PCR product was mixed with 16 μl of hybridization solution from a kit (Nisshinbo). Then denatured PCR product was mounted on a microarray with cover film (Nippon Genetics) and hybridized at 37°C for 2 hours in a hybridization cassette (TeleChem International Inc.). The cover film was removed in 0.2 × SSC after hybridization. The

slide was washed for 5 minutes in the same solution at 37°C. Hybridization signal was visualized using the conventional ABC method for coloring. Conjugate solution of streptavidin and horseradish peroxidase, 1.4 ml, prepared from the above kit according to the manufacturer's instruction, was mounted on the microarray and incubated at room temperature for 30 minutes. The slide was washed twice with coloring buffer from the kit (Tris-HCl, pH 8.0, NaCl and Tween 20) at room temperature for 5 minutes each. Coloring solution of tetramethylbenzidine, 1.4 ml, prepared from the kit according to the manufacturer's instruction, was mounted on the slide, which was incubated at room temperature for 30 minutes. The slide was rinsed in distilled water to end the coloring reaction, dried, and then scanned by a GT-8700F scanner (Seiko Epson Corp.) for analysis of the signal intensity on a computer.

Results

Each DNA microarray on a CarboStation slide contained 38 kinds of capture and reference oligonucleotides, as shown in Table 1. Under the condition employed herein, the two PCR fragments spanning the 5′ half of the mtDNA control region containing all polymorphic sites (Sato et al., 2001, 2004) specifically hybridized with the arrayed oligomers of examined sizes according to their nucleotide sequences, whereas the single PCR fragment encompassing that region often failed to exhibit a reproducible hybridization pattern (data not shown). This suggests that the larger fragment might be prevented from hybridizing with the arrayed oligomers owing to its possible secondary structure.

Figure 1 shows an example of hybridization signals detected on the DNA microarray with the above two fragments as a probe. The oligomer spots with intense hybridization signals were thought to contain perfectly matched sequence with a single nucleotide variation in the PCR fragments, whereas those with faint or no signals were thought to have no such sequence homology in the probe. As shown in Figure 1, transition of T to C and A to G at variable sites 10 and 42 discriminates haplotype A-1 from A-2 and A-3, and transversion of A to T and C to G at variable sites 57 and 154 discriminated haplotype A-1 from C-2 and B-3, respectively. Insertion of A at variable site 96 distinguished A-1 from A-8. Thus the present DNA microarray accurately detected all nucleotide sequence variations previously identified in the target region, which

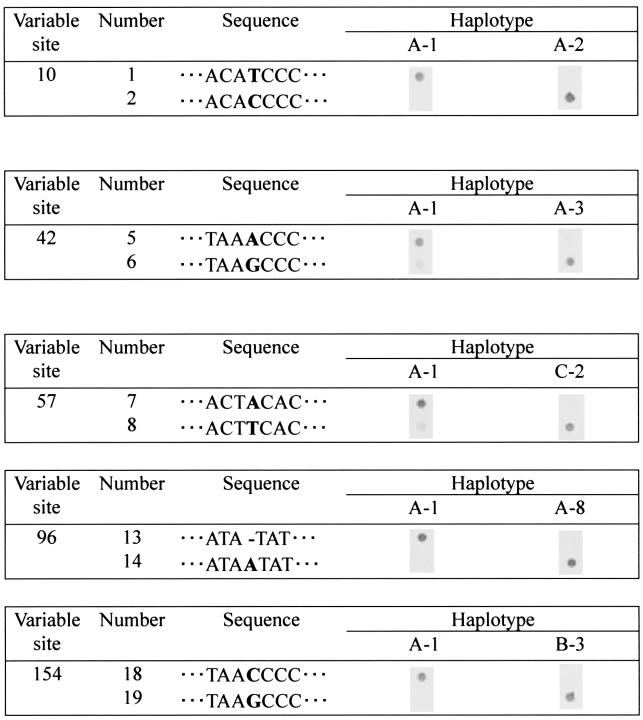

Figure 1. DNA microarray detection of representative polymorphic sites in 5′ half of mtDNA control region sequence of chum salmon. The number of variable sites indicates nucleotide position from the 5′ end of the mtDNA control region. Code number and polymorphic nucleotide (in boldface) of each sequence of immobilized oligomers are the same as those shown in Table 1.

identified a total of 30 haplotypes (Sato et al., 2001, 2004). Moreover, direct nucleotide sequencing of the same region in 37 chum salmon samples confirmed the results of DNA microarray analysis (data not shown).

Discussion

The present DNA microarray hybridization analysis successfully detected single nucleotide mutations including base substitutions and indels, thereby identifying the 20 previously described polymorphic sites of the 5′ half of the mtDNA control region of chum salmon, which defined 30 haplotypes (Sato et al., 2001, 2004). Our findings suggest that the present DNA microarray analysis will become a potential substitute for conventional sequence analysis in the detection of sequence polymorphism in both nuclear and mitochondrial DNA.

It is conceivable that the length of arrayed oligomers depends primarily on their sequence characteristics, such as the content and repetition of each nucleotide. These will

influence binding affinity—i.e. annealing temperature—in hybridization. The size of arrayed oligomers (Table 1) and hybridization temperature used herein were sufficient to obtain the specific hybridization pattern as shown in Figure 1. Higher hybridization stringency with stable results may also be available using the present protocol, since specific hybridization was obtained even at 43°C (data not shown). Failure of a longer probe to achieve stable hybridization also suggests that the length of PCR product is another important factor affecting hybridization, although such a failure could also be attributed to the sequence characteristics of the probe, possibly causing secondary structure as mentioned earlier. Such a secondary structural failure could occur with the arrayed oligomer sequence as well. This may be circumvented by reducing binding affinity in hybridization with substitution of a spacer nucleotide for an arbitrary one unrelated to sequence polymorphism. All these factors possibly affecting DNA microarray hybridization should be considered when designing the capture oligomers and probes.

Besides the technical merits of microarray preparation, the short time required for the present microarray analysis deserves emphasis. Hybridization, washing, signal visualization, and sequence typing can be completed within 4 hours. Special equipment such as a signal reader and image processor for fluorescent detection is not needed for the present method. Coloring of hybridization signals by the conventional ABC method actually makes it possible to optically determine mtDNA haplotypes even without scanner-assisted computation of signal intensity.

The features mentioned above thus indicate that the present DNA microarray method may become a means for GSI of chum salmon and other Pacific salmon species, providing rapid and accurate estimation of the origin of stocks. The developed method without any specialized laboratory equipment will be available for use in the field or on ships for commercial fisheries. In fact, mtDNA haplotypes of about 1000 chum salmon were determined during a research cruise sponsored by the Fisheries Agency, Japan, in the Bering Sea last September (Moriya et al., manuscript in preparation). The results of DNA microarray analysis on mixed ocean stocks of chum salmon will be published elsewhere.

Acknowledgments

This study was supported in part by Grants-in-aid from the fisheries Agency of Japan, Northern Advancement Center for Science and technology, Hokkaido, and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Abe, S., Sato, S., Kojima, H., Ando, J., Ando, H., Wilmot, R.L., Seeb, L.W., Efremov, V., LeClair, L., Buchholtz, W., Jin, D.-H., Urawa, S., Kaeriyama, M., and Urano, A. (2002). Development of molecular markers for genetic stock identification of chum salmon. *Fish Sci* 68(Suppl):353–356.

Brown, W.M., George, M. Jr., and Wilson, A.C. (1979). Rapid evolution of animal mitochondrial DNA. *Proc Natl Acad Sci U S A* 76:1967–1971.

Ferguson, A., Taggart, J.B., Prodohl, P.A., McMeel, O., Thompson, C., Stone, C., McGinnity, P., and Hynes, R.A. (1995). The application of molecular markers to the stocking and conservation of fish populations, with special reference to *Salmo*. *J Fish Biol* 47(Suppl A):103–126.

Hacia, J.G. (1999). Resequencing and mutational analysis using oligonucleotide microarrays. *Nat Genet* 21:42–47.

Lander, E.S. (1999). Array of hope. *Nat Genet* 21:3–4.

Lipshutz, R.J., Fodor, S.P.A., Gingeras, T.R., and Lockhart, D.J. (1999). High density synthetic oligonucleotide arrays. *Nat Genet* 21:20–24.

Sato, S., Ando, J., Ando, H., Urawa, S., Urano, A., and Abe, S. (2001). Genetic variation among Japanese populations of chum salmon inferred from the nucleotide sequences of the mitochondrial DNA control region. *Zool Sci* 18:99–106.

Sato, S., Kojima, H., Ando, J., Ando, H., Wilmot, R.L., Seeb, L.W., Efremov, V., LeClair, L., Buchholz, W., Jin, D.-H., Urawa, S., Kaeriyama, M., Urano, A., and Abe, S. (2004). Genetic population structure of chum salmon in the Pacific Rim inferred from mitochondrial DNA sequence variation. *Environ Biol Fish* 69:37–50.

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467–470.