NPAFC
Doc. 617
Rev.

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Submitted to the

NORTH PACIFIC ANADROMOUS FISH COMMISSION

by

JAPAN

October 2002

THIS PAPER MAY BE CITED IN THE FOLLOWING MANNER:
Shogo MORIYA, Syuiti ABE, Shigehiko URAWA, Osamu SUZUKI, Akihisa URANO. 2002. DNA microarray for rapid detection of mitochondrial DNA haplotypes of chum salmon. (NPAFC Doc. 617) 8 pages. Research and Development Center, Nisshinbo Industries, Inc. 1-2-3, Onodai, Midori-ku, Chiba, 267-0056, Japan

DNA microarray for rapid detection of mitochondrial DNA haplotypes of chum salmon

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ABSTRACT

We developed oligonucleotide (DNA) microarray hybridization method for rapid and accurate detection of nucleotide sequence variations in previously identified 20 variable nucleotide sites in about 500 bp 5' half of the control region of mitochondrial (mt) DNA of chum salmon (*Oncorhynchus keta*) for genetic stock identification (GSI). The method includes; 1) immobilization of synthesized 14 to 31 mer oligonucleotides containing the variable nucleotide on slide glass pre-coated with Poly-carbodiimide resin, 2) two-hour hybridization with DNA microarray of biotinylated PCR fragments spanning the 5' variable portion and subsequent short washing, and 3) visualization of hybridization signals colored by conventional ABC method and comparison of scanner-taking signal image on a computer. All the process excluding DNA extraction was completed within eight hours. The obtained DNA microarray could detect all the sequence variations defining a total of 30 haplotypes as revealed previously by nucleotide sequence analysis. The present DNA microarray method will therefore become a practical means for chum salmon GSI to be available in field or on board.

INTRODUCTION

Recently developed molecular techniques are expected to provide a powerful means with an increased accuracy and resolution to reveal genetic variation in salmon populations (Ferguson et al. 1995). Because of the higher sequence variability than single copy nuclear genes (Brown et al., 1979) and clonal haploid inheritance, analysis of mitochondrial (mt) DNA has received a considerable attention in genetic stock identification (GSI) of Pacific salmon species. However, such an analysis, mostly based on the restriction fragment length polymorphisms (RFLPs), revealed a low level of mtDNA sequence variation (Wilson et al. 1987, Cronin et al. 1993, Park et al., 1993, Bickham et al. 1995), providing the estimates of genetic divergence similar to those obtained from allozyme analyses (Seeb and Crane, 1999).

Recently, we have detected greater variation in the mtDNA control region of chum salmon (*Oncorhynchus keta*) by nucleotide sequence analysis than the variation observed by the previous RFLP analyses (Sato et al. 2001, Abe et al. 2002). 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 2,000 individuals from 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. in press). These findings suggest an increased potential for mtDNA sequence analysis to provide better estimation of stock composition in mixed ocean populations of chum salmon. 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 need a large number of samples.

In place of conventional sequence analysis using polyacrylamide gel electrophoresis or capillary electrophoresis, oligonucleotide (DNA) microarray-based hybridization analysis was suggested to be a promising new technology, potentially allowing rapid and cost-effective screening of all possible mutations and sequence variations in genomic DNA (Hacia 1999, Landa 1999). In the present study, we attempted to develop a rapid and accurate method to detect mtDNA haplotypes of chum salmon for GSI using DNA microarray based on reported sequence variations in the control region (Sato et al. 2001, in press). The obtained DNA microarray detected all the 30 haplotype sequences which were revealed in the previous nucleotide sequence analysis (Sato et al. 2001, in press). The present DNA microarray method will become a practical means for chum salmon GSI to be available in field or on board.

MATERIALS AND METHODS

DNA extraction

About 50?l of liver homogenate from 37 individuals including 34 known and 3 unknown for mtDNA haplotypes was added to 500?l sodium tris EDTA buffer (0.1M NaCl, 10mM Tris-HCl, and 1mM EDTA, pH8.0) containing 500?g/ml proteinase K and 0.5% SDS, and incubated at 37°C overnight. DNA was extracted with a mixture of phenol (250?l) and 24:1 chloroform:isoamylalcohol (250?l) three times and then twice with 500?l of 24:1 chloroform:isoamylalcohol alone. DNA in aqueous phase was recovered by ethanol precipitation, dried in air, and dissolved in TE (10mM Tris-HCl, 1mM EDTA, pH7.5)

PCR amplification

PCR primers were designed for amplification of two fragments spanning about 500 bp 5' variable portion of the mtDNA control region that included all the variable sites. Identified previously (Sato et al. 2001, in press). These two fragments were amplified separately in a 50?l of reaction mixture containing 25-100ng of template DNA, 10mM Tris-HCl (pH8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.25mM each dNTP, 1U *Taq* DNA polymerase (TaKaRa) 1?M of forward and reverse primers. The latter was biotinylated for simultaneous labeling with PCR amplification. The condition of PCR using a DNA engine (MJ research) was as follows; preheating at 95°C for 3min, followed by 40 cycles of denaturation at 95°C for 1min, annealing at 45°C for 30 sec, and extension at 72°C for 30 sec, with post-cycling extensions at 72°C for 3min.

DNA microarray preparation

Oligonucleotides for microarray were synthesized at Nisshinbo Industries, Tokyo. The size of oligonucleotides was 14-31 mer, and each oligomer contained the previously identified single nucleotide variations such as base substitutions and indels at the center (Table 1). Immobilization of DNA oligomers to CarboStation^{R} slides (Nisshinbo) was according to the manufacturer's instruction. The CarboStation^{R} slides for DNA microarray was originally developed by Nisshinbo Industries. DNA oligomers can be immobilized covalently on the CarboStation^{R} since its surface is uniformly coated with Poly-carbodiimide resin. Thus, each DNA microarray on a CarboStation^{R} slide contained all the possible 20 mutation sites to detect each of 30 haplotype sequences previously identified (Sato et al., in press). Each oligomer spot in a microarray was coded as showin in Table 1.

Hybridization and signal detection

Two ?l each of denatured PCR reaction mixture was mixed with 16?l of hybridization buffer (Nisshinbo), mounted on a microarray with cover glass, and hybridized at 37°C for two hours. After hybridization, the slide was washed in a washing buffer (Nisshinbo) at 37°C for 5min. Hybridization signal was visualized using

conventional ABC method for coloring. Conjugate solution, 1.4ml, prepared from a kit (Nisshinbo) according to the manufacturer's instruction, was mounted on the microarray and incubated at room temperature for 30min. The slide was washed twice by coloring buffer (Nisshinbo) at room temperature for 5min each. Coloring solution, 1.4ml, prepared from the above kit according to the manufacturer's instruction, was mounted on the microarray and incubated at room temperature for 30 min. The microarray was rinsed in distilled water, dried, and then scanned by GT-8700F scanner (EPSON) for analyzing the signal intensity on a computer.

RESULTS

Fig. 1 shows an example of hybridization signals detected on DNA microarray. The oligomer spots with strong hybridization signals were expected to contain perfectly matched single nucleotide variation in PCR fragments, whereas those with faint or no signals were thought to have no such sequence homology in PCR products. In fact, this was confirmed by blind comparison of the results of microarray hybridization with the previously identified nucleotide sequences in several chum salmon sample (data not shown). Therefore, oligomer spots with stronger hybridization signals were taken as positive sites, and their code number was aligned to determine corresponding haplotypes. For example, positive spots shown in Fig 1 were 1, 3, 6, 7, 9, 11, 13, 15, 18, 20, 22, 24, 26, 28, 30, 33, 35, and 37, which was correspondent to haplotype A-3 in Table 2.

As shown in Table 2, DNA microarray hybridization analysis detected all the single nucleotide mutations as previously identified (Sato et al 2001, in press). Thus, all the 37 individuals examined showed concordance of the results from microarray hybridization and conventional sequence analysis (Table 3).

DISCUSSION

The present DNA microarray hybridization analysis successfully detected nucleotide sequence variations occurred in 20 variable sites of the 5' half of the mtDNA control region of chum salmon as previously identified (Sato et al. 2001, in press). Our findings suggest that DNA microarray analysis is a potential substitute for conventional sequence analysis when detecting mutations and sequence variations in both nuclear and mitochondrial DNA.

Immobilization of synthetic DNA oligomers on Poly-carbodiimide resin pre-coated glass slide has an advantage over light-directed oligonucleotide microarray preparation (Lipshutz et al. 1999), although the latter makes it possible to immobilize high density synthetic oligomer arrays on a chip. Poly-carbodiimide resin facilitates covalent binding of synthetic oligomers to slide glass without linkers, so that microarrays can be prepared arbitrarily using oligomers with desired size and sequence. This will greatly reduce the cost of microarray preparation, allowing incorporation of a number of fish samples in GSI.

Besides the technical merit of microarray preparation, quick performance of the present microarray analysis would deserve emphasis. Experimental process including hybridization, washing, signal visualization, and sequence typing can be completed within eight hours. No particular equipment such as signal reader and image processor is needed for the present analysis. Coloring of hybridization signals by conventional ABC method actually makes it possible to optically determine mtDNA haplotypes even without scanner-assisted computation of signal intensity.

Features mentioned above thus indicate that the present DNA microarray method may become a potential means for GSI of chum salmon and other Pacific salmon species, providing rapid and accurate estimation of the origin of stocks. Practically, the developed method without any specialized laboratory equipment will be available in field or on ships for commercial fisheries. Confirmation of such availability is now ongoing in our laboratories using a research vessel of Fisheries Agency of Japan.

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.

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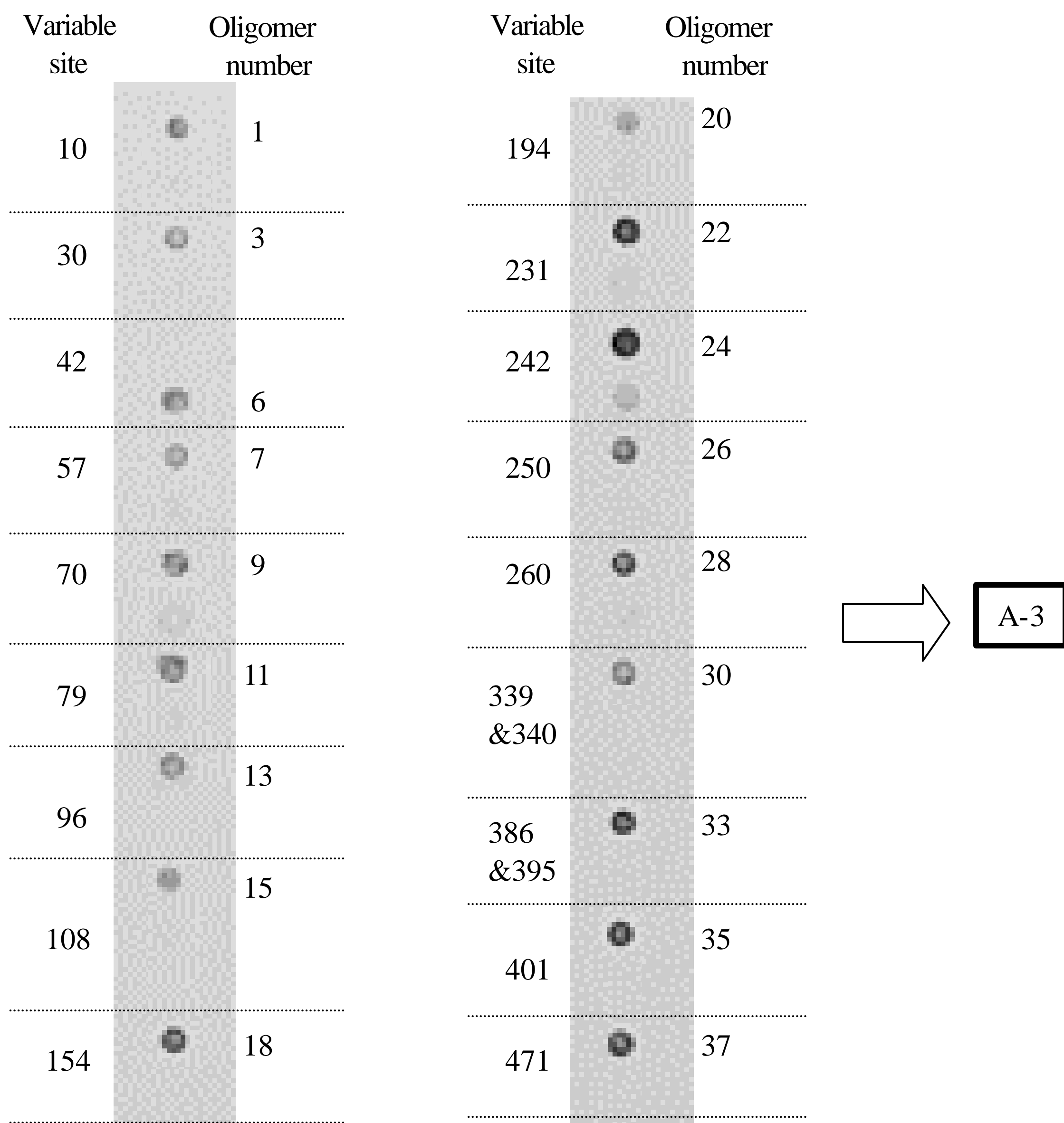

Figure 1. Detection of hybridization signals

Table 1. Oligomers immobilized to slide glass. Vairable nucleotide is shown in red. A:adenine, T:thymine, G:guanine, C:cytosine, –:delation. Number at variable site shows positions from the 5’ end of the mtDNA control region of chum salmon. Code number shows the number of synthesized oligomer.

Variable site	Number	sequence	Variable site	Number	sequence
10	1	???ACATCCC???	194	20	???AGCAAAA???
	2	???ACACCCC???		21	???AGCTAAA???
30	3	???TGCTATA???	231	22	???AACTGAT???
	4	???TGCCATA???		23	???AACCGAT???
42	5	???TAAACCC???	242	24	???TGCCGCA???
	6	???TAAGCCC???		25	???TGCTGCA???
57	7	???ACTACAC???	250	26	???CAATAAA???
	8	???ACTTCAC???		27	???CAACAAA???
70	9	???TAATATT???	260	28	???CCAACTA???
	10	???TAACATT???		29	???CCAGCTA???
79	11	???ATATTAT???	339	30	???ATATCAGT???
	12	???ATACTAT???	& 340	31	???ATAACAGT???
96	13	???ATA–TAT???		32	???ATAATAGT???
	14	???ATAATAT???	386	33	???AGGTC??CTCGTG???
108	15	???TGCTCGT???	& 395	34	???AG–TC??CTAGTG???
	16	???TGCACGT???	401	35	???GAATTAT???
	17	???TGCCCGT???		36	???GAACTAT???
154	18	???TAACCCC???	471	37	???TAAAGCA???
	19	???TAAGCCC???		38	???TAACGCA???

Table 2. Oligonucleotide microarray detection of 20 variable sites in the 5' half of the mtDNA control region of chum salmon. Representative siganl profile of positive oligomer spots was shown for 16 of previously identified 30 mtDNA haplotypes.

Selected oligomer number	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4
	5	6	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	7	7	7	7	7	7	7	7	7	7	7	7	7	8	7	7
	9	9	9	9	9	9	9	9	9	9	9	9	9	9	10	9
	11	11	11	11	11	12	11	11	11	11	11	11	11	11	11	11
	13	13	13	13	14	13	13	13	13	13	13	13	13	13	13	13
	15	15	15	15	15	15	17	15	15	15	15	15	15	15	15	16
	18	18	18	18	18	19	19	19	19	19	19	19	19	18	18	18
	20	20	21	20	20	20	20	20	20	20	20	20	20	20	20	20
	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22
	24	24	24	24	24	24	24	25	24	24	24	24	24	24	24	24
	26	26	26	26	26	26	26	26	27	26	26	26	26	26	26	26
	28	28	28	28	28	28	28	28	28	29	28	28	28	28	28	28
	30	30	30	30	30	30	30	30	30	30	30	32	31	30	30	30
	33	33	33	33	33	34	34	34	34	34	34	34	34	33	33	33
	35	35	35	35	35	35	35	35	35	35	35	35	36	35	35	35
	37	37	37	37	37	37	37	37	37	37	38	37	37	37	37	37
Decided haplotype	A-2	A-3	A-5	A-6	A-8	B-7	B-8	B-10	B-11	B-12	B-15	B-16	B-17	C-2	C-3	C-4

Table 3. Comparison of the results between DNA microarray hybridization and sequencing analysis

sample number	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
haplotype	A-1	A-1	A-2	A-3	A-4	A-4	A-5	A-6	A-6	A-7
concordance with sequencing method	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**

sample number	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)
haplotype	A-8	A-8	B-1	B-3	B-3	B-4	B-5	B-5	B-6	B-7
concordance with sequencing method	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**

sample number	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
haplotype	B-8	B-9	B-9	B-10	B-11	B-12	B-13	B-14	B-14	B-15
concordance with sequencing method	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**

sample number	(31)	(32)	(33)	(34)	(35)	(36)	(37)
haplotype	B-16	B-17	C-1	C-1	C-2	C-3	C-4
concordance with sequencing method	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**