Application of PCR Primer Pairs from Rainbow Trout to Detect Polymorphisms of CA Repeat DNA Loci in Five Confamilial Species

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Dinucleotide repeat polymorphisms are widely used as markers for genetic analysis in mammalian genomes because of their abundance and high variability among individuals. We have previously isolated informative markers for genetic studies of rainbow trout. In fisheries science, many species need to be analyzed. Therefore, heterologous applications of DNA markers designed in one species can be useful. The sequences flanking a dinucleotide repeat are often conserved among closely related species. We thus wanted to determine whether DNA markers isolated from the rainbow trout genome could be used to detect dinucleotide repeat polymorphisms in five other species of salmonid. Five sets of primer pairs that flank rainbow trout microsatellites and that produced polymorphic PCR products, were tested with template DNA from five species. Specific PCR products were obtained in all cases. Four out of five DNA markers could detect polymorphism in all species and all five DNA markers could detect polymorphism in at least one species. In many cases, these markers showed large numbers of alleles and high heterozygosity values in other salmonids such as rainbow trout. Our results show that DNA markers isolated from rainbow trout are also useful in other salmonids for fisheries sciences such as population studies and genetic linkage analysis.

Key words: dinucleotide repeat polymorphism, genetic mapping, linkage analysis, rainbow trout, microsatellite

Introduction

The dinucleotide repeat sequences (dG–dT)_n and (dC–dA)_n are widely interspersed in eukaryotic genomes,^{1–3)} and are often polymorphic in mammalian species.^{4–7)} Polymorphic loci containing (dG–dT)_n repeats tend to be more informative than conventional markers (RFLPs; restriction fragment length polymorphisms).⁸⁾ VNTR (variable number of tandem repeat) markers often reveal high heterozygosity, but are not evenly distributed throughout the genome.⁹⁾ Dinucleotide repeat polymorphisms have proven to be evenly distributed throughout the genomes.^{4–8,10–12)} For this reason, genetic markers based on (dG–dT) repeat polymorphisms are widely used for genomic mapping, linkage analysis of genetic diseases and population studies.^{13–16)}

To identify quantitative traits of economic importance to fisheries, e.g. disease resistance, we are attempting to isolate DNA markers from the rainbow trout genome.^{17–21)} In fisheries sciences, many species need to be analyzed. If DNA markers isolated from one species can be used for different species, they must be a more profitable tool for genome analysis in fisheries sciences. In mammals, the locations of dinucleotide microsatellites are often conserved among closely related species, and can serve for detecting polymorphisms in several different species.^{16,22)} In fisheries science, a trinucleotide microsatellite cloned from the rainbow trout genome was employed in different species for DNA fingerprinting.²³⁾ To investigate whether DNA mark-

ers based on dinucleotide repeat polymorphisms of rainbow trout can be used in other salmonids, we attempted to detect polymorphisms in five species using DNA markers of rainbow trout.

Materials and Methods

Fish Samples

Eight individuals of each of the following species were sampled: coho salmon *Oncorhynchus kisutch* were obtained from Miyagi Prefectural Freshwater Experimental Station, Japanese char *Salvelinus pluvius* from Miyagi Prefectural Freshwater Experimental Station and Gifu, Shizuoka, and Aichi Prefectural Fisheries Experimental Station, brook trout *Salvelinus fontinalis* from Shizuoka Prefectural Fisheries Experimental Station, amago salmon *Oncorhynchus macrostomus* from Gifu, Shizuoka, and Aichi Prefectural Fisheries Experimental Station, and yamame salmon *Oncorhynchus masou* from Miyagi Prefectural Freshwater Experimental Station and Gifu Prefectural Fisheries Experimental Station. Genomic DNA was extracted from whole blood cells by the method of Strauss.²⁴⁾

DNA Markers

The five DNA markers (FGT1, FGT2, FGT3, FGT4, and FGT5)^{17–21)} used in this study are listed in Table 1. The DNA markers were isolated as described by Sakamoto *et al.*¹⁷⁾

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Table 1. CA repeat DNA markers of rainbow trout used in this study

Marker	Primer sequence (5'-3')	Reference
FGT1	AGATTACCCAGCCAGGTAG CATAGTCTGAACAGGGACAG	(17)
FGT2	GTGTGGAACCCAACAGGC GAAACACGTCTGGGGTCAGT	(18)
FGT3	CAAGAAATTTGTGGAGCGG GAAGCCCTGTTTGACTTTTAGC	(19)
FGT4	CTCACAACAGCACACGCAC CATTTTCAGGTTTGGCGC	(20)
FGT5	TCCTTTTCTCCCTTTCTTTCC TCCAGCCAGACACACACG	(21)

PCR and Detection of Polymorphisms

The PCR reaction was performed in a 25 μ l reaction volume containing 20 pM each of one unlabeled primer and one primer end-labeled with [γ - ^{32} P] ATP using T4 polynucleotide kinase, plus 400 μ M of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.15 mM MgCl₂, 1 U *Taq* DNA polymerase (Takara) and 0.2 μ g template DNA. The PCR program consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles of 55°C for 1 min, 72°C for 1 min, 95°C for 30 s, and a final extension of 3 min at 72°C. PCR products were mixed with equal volumes of formamide loading buffer (0.3% xylene cyanol, 0.3% bromophenol blue in formamide) and denatured at 75°C for 5 min, prior to electrophoresis in a 6% polyacrylamide 7 M urea gel. The gel was dried and autoradiographed at -80°C overnight.

Results

To detect length-polymorphisms in (dG-dT)_n repeat loci, DNA samples from eight individuals in each of the five species were amplified by PCR and then separated by an acrylamide gel containing 7 M urea. Specific PCR products were observed in all cases (25/25; 100%). Four out of five DNA markers designed for use in rainbow trout could detect length-polymorphisms in all species and all five DNA markers could detect polymorphism in at least one species. Twenty-one of 25 tested cases (84%) showed polymorphisms. The results are summarized in Table 2. Examples of PCR products amplified by FGT3 are shown in Fig. 1. The polymorphism could be detected only in Japanese char with the DNA marker FGT4 (Fig. 2). To investigate whether dinucleotide repeat sequences were included in PCR products amplified by these PCR primer pairs, the PCR products were analyzed by Southern blot hybridization with a [γ - ^{32} P] end-labeled (dG-dT)₁₀

Table 2. Use of heterologous PCR primer pairs in different species

Species	No specific product	Specific and monomorphic	Specific and polymorphic
Coho salmon	0	1	4
Japanese char	0	0	5
Brook trout	0	1	4
Amago salmon	0	1	4
Yamame salmon	0	1	4

Fig. 1. Autoradiograph of a denaturing polyacrylamide gel showing PCR products in different salmonid species by PCR primer pairs of FGT3 from rainbow trout.

Lanes 1-8, coho salmon; lanes 9-16, Japanese char; lanes 17-24, brook trout; lanes 25-32, amago salmon; lanes 33-40, yamame salmon. The molecular size in base pairs is indicated on the right.

Fig. 2. Autoradiograph of a denaturing polyacrylamide gel showing PCR products in different salmonid species by PCR primer pairs of FGT4 from rainbow trout.

Lanes 1-8, coho salmon; lanes 9-16, Japanese char; lanes 17-24, brook trout; lanes 25-32, amago salmon; lanes 33-40, yamame salmon. The molecular size in base pairs is indicated on the right.

Table 3. Observed heterozygosity and total allelic fragment data in different species for the five CA repeat DNA loci of rainbow trout

Marker	Species					
	Rainbow trout ^{*1}	Coho salmon	Japanese char	Brook trout	Amago salmon	Yamame salmon
FGT1	0.666(7) ^{*2}	0.625(6)	0.250(3)	0.250(2)	0.375(7)	0.625(5)
FGT2	0.600(4)	0.500(7)	0.750(6)	0.375(3)	0.00(4)	0.500(6)
FGT3	0.800(8)	0.500(5)	0.625(5)	0.500(4)	0.625(3)	0.750(4)
FGT4	0.600(8)	np ^{*3}	0.125(3)	np	np	np
FGT5	0.700(3)	0.500(2)	0.750(6)	0.750(6)	0.625(4)	0.625(7)

^{*1} Heterozygosities of rainbow trout were reported previously.¹⁷⁻²¹⁾

^{*2} Observed heterozygosity and the total number of allelic fragments in the parentheses.

^{*3} np: no polymorphism.

oligonucleotide probe. In all cases, PCR products could be detected and only the signals of PCR products amplified by primer pairs of FGT4 were very weak in all species (data not shown). In several of the species as well as in rainbow trout, these markers revealed large numbers of alleles and high heterozygosity values. The average heterozygosity

ty value for all cases in which polymorphisms could be detected was 50.1%. The results are summarized in Table 3.

Discussion

The preceding results have shown that DNA markers based on dinucleotide repeat polymorphisms in rainbow trout are useful for other salmonids.

In humans and other mammals, DNA markers based on RFLPs and VNTR have significantly contributed to the development of genetic linkage maps,²⁵⁾ and to the identification of genes associated with genetic diseases such as familial adenomatous polyposis coli,²⁶⁾ cystic fibrosis²⁷⁾ and neurofibromatosis type 1,²⁸⁻³⁰⁾ and to the detection of tumor suppressor genes.³¹⁾ Usually, however, only 30–40% of individuals are informative for conventional RFLPs in genetic linkage studies. VNTR markers often reveal high heterozygosity, but tend to be distributed in the telomeric regions of human chromosomes.⁹⁾ On the other hand, dinucleotide repeat polymorphisms have been reported to be highly informative and evenly distributed throughout the genomes.^{4-8,10-12)} Therefore, DNA markers based on dinucleotide repeat polymorphisms have been more widely used than other kinds of DNA markers. PCR-based microsatellite DNA markers could facilitate the construction of a high-resolution linkage map from small amounts of DNA. However, the main problem with microsatellite DNA marker analysis is that a suitable set of primers has to be developed for each species, which can be laborious, expensive and time consuming. In mammals, the conservation of dinucleotide microsatellites was close enough to allow PCR primers designed for use in one species to be used to analyze length-polymorphisms in another.^{16,22)} In fish, a trinucleotide (purine-purine-pyrimidine) repeat cloned from the rainbow trout genome was tested in different species for DNA fingerprinting and the results suggested that the trinucleotide repeat locus was conserved and remained polymorphic in other salmonid species.²³⁾ Therefore, we have analyzed the usefulness of rainbow trout DNA markers on other salmonids. The DNA markers, which are based on dinucleotide repeat polymorphisms, can detect a single locus in rainbow trout and show high heterozygosity among individual rainbow trout.¹⁷⁾ As a result, each of the five DNA markers designed for use in rainbow trout could detect length-polymorphisms in other salmonids. Four of these markers could detect length-polymorphisms in all species tested in this study. For several species, as well as rainbow trout, these markers showed large numbers of alleles and high heterozygosity values. Our results imply that DNA markers of rainbow trout could be employed for genetic and population studies in salmonid species. The use of heterologous primers will significantly reduce the cost for developing markers for salmonid species.

The evolution of salmonid species genomes was investigated using tRNA-derived retrotransposons.³²⁾ To analyze the genome organization and evolution, the comparative mapping approach has been performed in man, cattle, rat and mouse using DNA markers and homologous genes.^{33,34)} Our results suggested that rainbow trout DNA markers might also allow comparative mapping among salmonid genomes. The isolation of DNA markers based on dinucleotide repeat polymorphisms from the rainbow

trout genome should also contribute to the linkage analysis of genes related to mutant phenotypes and to the linkage analysis of genes related to traits with economic significance in other salmonids.

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References

- 1) R. Miesfeld, M. Krystal, and N. Arnheim: A member of a new repeated sequence family which is conserved throughout eucaryotic evolution is found between the human δ and β globin genes. *Nucleic Acids Res.*, **9**, 5931–5947 (1981).
- 2) H. Hamada and T. Kakunaga: Potential Z-DNA forming sequences are highly dispersed in the human genome. *Nature*, **298**, 396–398 (1982).
- 3) R. L. Stallings, A. F. Ford, D. Nelson, D. C. Torney, C. E. Hildebrand, and R. K. Moyzis: Evolution and distribution of (GT)_n repetitive sequences in mammalian genomes. *Genomics*, **10**, 807–815 (1991).
- 4) M. Litt and J. A. Luty: A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.*, **44**, 397–401 (1989).
- 5) D. Tautz: Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.*, **17**, 6463–6471 (1989).
- 6) J. L. Weber and P. E. May: Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.*, **44**, 388–396 (1989).
- 7) R. Fries, A. Eggen, and G. Stranzinger: The bovine genome contains polymorphic microsatellites. *Genomics*, **8**, 403–406 (1990).
- 8) J. L. Weber: Human DNA polymorphisms based on length variations in simple-sequence tandem repeats, in "Genome Analysis" (ed. by K. E. Davies and S. M. Tilghman), Vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992, pp. 159–181.
- 9) N. J. Royle, R. E. Charkson, Z. Wong, and A. J. Jeffreys: Clustering of hypervariable minisatellites in the proterminal regions of human autosomes. *Genomics*, **3**, 352–360 (1988).
- 10) J. S. Beckmann and J. L. Weber: Survey of human and rat microsatellites. *Genomics*, **12**, 627–631 (1992).
- 11) J. M. Love, A. M. Knight, M. A. Mcleer, and J. A. Tod: Towards construction of a high resolution map of the mouse genome using PCR-analysed microsatellites. *Nucleic Acids Res.*, **18**, 4123–4130 (1990).
- 12) H. Hamada, M. G. Petrino, and T. Kakunaga: A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 6465–6469 (1982).
- 13) M. Georges, A. B. Diets, A. Mishra, D. Nielsen, L. S. Sargeant, A. Scorensen, M. R. Steele, X. Zhao, H. Leipold, J. E. Womack, and M. Lathrop: Microsatellite mapping of the gene causing weaver disease in cattle will allow the study of an associated quantitative trait locus. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 1058–1062 (1993).
- 14) D. J. Kwiatkowski, E. P. Henske, K. Weimer, L. Ozelius, J. F. Gusella, and J. Haines: Construction of GT polymorphism map of human 9q. *Genomics*, **12**, 229–240 (1992).
- 15) J. M. Hall, L. Friedman, C. Guenther, M. K. Lee, J. L. Weber, D. M. Black, and M.-C. King: Closing in on a breast cancer gene on chromosome 17q. *Am. J. Hum. Genet.*, **50**, 1235–1242 (1992).
- 16) C. Schlotterer, B. Amos, and D. Tautz: Conservation of polymorphic sample sequence loci in cetacean species. *Nature*, **354**, 63–65 (1991).

- 17) T. Sakamoto, N. Okamoto, Y. Ikeda, Y. Nakamura, and T. Sato: Dinucleotide-repeat polymorphism in DNA of rainbow trout, *Oncorhynchus mykiss*, and its application in fisheries science. *J. Fish Biol.*, **44**, 1093–1096 (1994).
- 18) T. Sakamoto, N. Okamoto, and Y. Ikeda: Dinucleotide repeat polymorphism of rainbow trout, FGT2. *J. Anim. Sci.*, **72**, 2765 (1994).
- 19) T. Sakamoto, N. Okamoto, and Y. Ikeda: Dinucleotide repeat polymorphism of rainbow trout, FGT3. *J. Anim. Sci.*, **72**, 2766 (1994).
- 20) T. Sakamoto, N. Okamoto, and Y. Ikeda: Dinucleotide repeat polymorphism of rainbow trout, FGT4. *J. Anim. Sci.*, **72**, 2767 (1994).
- 21) T. Sakamoto, N. Okamoto, and Y. Ikeda: Dinucleotide repeat polymorphism of rainbow trout, FGT5. *J. Anim. Sci.*, **72**, 2768 (1994).
- 22) S. S. Moore, L. L. Sargeant, J. King, J. S. Mattick, M. Georges, and D. J. S. Hetzel: The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. *Genomics*, **10**, 654–660 (1991).
- 23) P. Bentzen, S. Harris, and M. Wright: Cloning of hypervariable minisatellite and simple sequence microsatellite repeats for DNA fingerprinting of important aquacultural species of salmonids and *Tilapia*, in "DNA Fingerprinting: Approaches and Applications" (ed. by T. T. Burke, G. Dolf, and R. Wolff), Birkhauser Verlag, Bern, 1991, pp. 243–262.
- 24) W. M. Strauss: Preparation of genomic DNA from mammalian tissue, in "Current Protocols" (ed. by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl), Vol. 1, John Wiley & Sons, New York, 1987, pp. 2.2.1–2.2.3.
- 25) K. Yamakawa, R. Morita, E. Takahashi, T. Hori, M. Lathrop, and Y. Nakamura: A genetic linkage map of 41 restriction fragment length polymorphism markers for human chromosome 3. *Genomics*, **11**, 565–572 (1991).
- 26) K. W. Kinzler, M. C. Nilbert, L.-K. Su, B. Vogelstein, T. M. Bryan, D. B. Levy, K. J. Smith, A. C. Preisinger, P. Hedge, D. McKechnie, P. Finnear, A. Markham, J. Groffen, M. S. Boguski, S. F. Altschul, A. Horii, H. Ando, Y. Miyoshi, Y. Miki, I. Nishisho, and Y. Nakamura: Identification of FAP locus genes from chromosome 5q21. *Science*, **253**, 661–665 (1991).
- 27) J. R. Riordan, J. M. Rommens, B.-S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, M. L. Drumm, M. C. Iannuzzi, F. C. Collins, and L.-C. Tsui: Identification of the cystic fibrosis gene; Cloning and characterization of complementary DNA. *Science*, **245**, 1066–1073 (1989).
- 28) D. Viskochil, A. M. Buchberg, G. Xu, R. M. Cawthon, J. Stevens, R. K. Woff, M. Culver, J. C. Carey, N. G. Copeland, N. A. Jenkins, R. White, and P. O'Connell: Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell*, **62**, 187–192 (1990).
- 29) R. M. Cawthon, R. Weiss, G. Xu, D. Viskochil, M. Culver, J. Stevens, M. Robertson, D. Dunn, R. Gesteland, P. O'Connell, and R. White: A major segment of the neurofibromatosis type 1 gene; cDNA sequence, genomic structure, and point mutations. *Cell*, **62**, 193–201 (1990).
- 30) M. R. Wallace, D. A. Marchuk, L. B. Andersen, R. Letcher, H. M. Odeh, A. M. Saulino, J. W. Fountain, A. Brereton, J. Nicholson, A. L. Mitchell, B. H. Brownstein, and F. C. Collins: Type 1 neurofibromatosis gene; Identification of a large transcript disrupted in three NF1 patients. *Science*, **249**, 181–186 (1990).
- 31) T. Sato, A. Tanigami, K. Yamakawa, F. Akiyama, F. Kasumi, G. Sakamoto, and Y. Nakamura: Allelotype of breast cancer; Cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.*, **50**, 5794–5799 (1991).
- 32) S. Murata, N. Takasaki, M. Saitoh, and N. Okada: Determination of the phylogenetic relationships among Pacific salmonids by using short interspersed elements (SINEs) as temporal landmarks of evolution. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 6995–6999 (1993).
- 33) N. Zhang and J. E. Womack: Synteny mapping in the bovine; Genes from human chromosome 5. *Genomics*, **14**, 126–130 (1992).
- 34) E. F. Remmers, E. A. Goldmuntz, H. Zha, P. Mathern, Y. Du, L. J. Crofford, and R. L. Wilder: Linkage map of nine loci defined by polymorphic DNA markers assigned to rat chromosome 13. *Genomics*, **18**, 277–282 (1993).