Cross-species amplification of microsatellite markers for the brown sole in the family Pleuronectidae

SANG-GYU KIM [a*] KAGAYAKI MORISHIMA and KATSUTOSHI ARAI

Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[*]Corresponding author: Tel.: (+82) 51-720-2449
Fax: (+82) 51-720-2456; E-mail: sgkim39@gmail.com

[a]Present address: Biotechnology Research Institute, National Fisheries Research & Development Institute, Busan 619-705, Korea

Abstract

The amplification of eight microsatellite loci previously developed and characterized in brown sole *Pleuronectes herzensteini* was attempted in 11 other flatfish species. Two loci *Phz6* and *Phz12* were amplified in all the species examined. Cross-species amplification was succeeded in eight loci of *Kareius bicoloratus* and *Pleuronectes yokohamae*, but in seven loci of *Microstomus achne, Pleuronectes punctatissimus* and *Pleuronectes schrenki*. Five to three loci could be amplified in other six species. In the three species selected, *K. bicoloratus*, *P. punctatissimus* and *P. yokohamae*, cross-amplified seven to eight loci exhibited polymorphisms comprising one to 22 alleles. Expected heterozygosity (*He*) ranged from 0.66 to 0.96 in *K. bicoloratus*, from 0.62 to 0.96 in *P. punctatissimus* and from 0.43 to 0.91 in *P. yokohamae*. Observed heterozygosity (*Ho*) ranged from 0.65 to 1.00 in *K. bicoloratus*, from 0.55 to 0.95 in *P. punctatissimus* and from 0.40 to 0.95 in *P. yokohamae*. The *Phz2, Phz3* and *Phz12* loci significantly deviated in certain or all the three species from Hardy-Weinberg equilibrium. The mean values of homology to the flanking-region sequences of brown sole were 93.7% in *K. bicoloratus*, 91.2% in *P. punctatissimus*, and 93.9% in *P. yokohamae*. These results suggest that microsatellite markers for brown sole are applicable for genetic studies in flatfish species including at least these three species.

Keywords: Cross-species amplification, *Kareius bicoloratus*, Microsatellites, *Pleuronectes punctatissimus*, *Pleuronectes yokohamae*

1. Introduction

Benthic flatfishes are important as inshore fisheries resources in Japan. In seven flatfish species (Japanese or olive flounder *Paralichthys olivaceus*; brown sole *Pleuronectes herzensteini*; cresthead flounder *Pleuronectes schrenki*; marbled sole *Pleuronectes yokohamae*; willowy flounder *Tanakius kitaharai*; barfin flounder *Verasper moseri*; and spotted halibut *Verasper variegatus*), artificial seeds have been produced in hatchery and released for stock enhancement. However, genetic studies of these flatfish species were not satisfactory to disclose influence of stocking on wild populations, except for a limited numbers of studies conducted in Japanese flounder [1-3] as well as barfin flounder [4,5].

Development of polymorphic molecular markers is a first step for genetic studies, but it is generally time-consuming to isolate and characterize DNA markers for each target species. Such tools have not been developed in most commercially important flatfish species. When cross-amplification of molecular markers previously developed in a certain species is possible in the other related species, we can promote genetic studies of target species more efficiently using markers developed in other species. In flatfish species, Ortega-Villaizan Romo et al. [6] reported the successful cross-species reaction of microsatellite DNA markers developed in barfin flounder *Verasper moseri* in a different but closely related species, spotted halibut *Verasper variegates*. Recently, cross amplification has been studied to develop universal primer sets which can act as many related species as possible. Liu et al. [7] reported the availabilities of microsatellite markers developed from an expressed sequence tag (EST) library of the olive flounder in other marine fishes, including turbot *Scophthalmus maximus*, sea perch *Lateolabrax japonicus*, red sea bream *Pagrus major*, black porgy *Sparus macrocephalus* and half-smooth tongue sole *Cynoglossus semilaevis*.

We have already isolated and characterized polymorphic microsatellite DNA markers in the brown sole, *Pleuronectes herzensteini* [8]. In the present study, cross-species amplification of these microsatellite loci was screened in a total of 11 flatfish species. Then, closely related three flatfish species, stone flounder *Kareius bicoloratus*, sand flounder *P. punctatissimus* and marbled sole *P. yokohamae* were selected for further studies, because all these species are commercially important in fishery industry in Hokkaido, Japan and marbled sole is a target species of artificial seed stocking. We attempted to amplify of eight microsatellite loci for brown sole. The homology was also examined in flanking regions (>100

bp) of microsatellite loci of other flatfish species, by which cross-amplification was possible.

2. Materials and methods

2-1. Fish samples

Two or one specimens of the 11 flatfish species (*Glyptocephalus stelleri*, *Hippoglossoides pinetorum*, *H. dubius*, *Kareius bicoloratus*, *Microstomus achne*, *Pleuronectes mochigarei*, *P. schrenki*, *P. yokohamae*, *P. punctatissimus*, *Tanakius kitaharai*, *Verasper moseri*) were obtained from the commercial market and then used for screening of cross amplification. Other specimens of *K. bicoloratus* and *P. punctatissimus* were caught by fishing in the coast of Kogane, Date city, Hokkaido, Japan. Marbled sole *P. yokohamae* specimens were caught by fishing in the coast of Toubetsu, Hokuto city, Hokkaido, Japan.

2-2. DNA extraction and PCR amplification

Samples were fixed in 99.9% ethanol, stored in 4 M-Urea buffer [9] and then subjected to extract DNA from fin-clip using the standard phenol-chloroform method [10].

Eight loci of microsatellite DNA markers (*Phz2*, *Phz3*, *Phz4*, *Phz6*, *Phz7*, *Phz8*, *Phz12* and *Phz14*) developed in brown sole [8] were cross-species amplified in DNA samples from different flatfish species. PCR was performed in 10 µl reaction mixture containing 50-100 ng DNA template. Reaction mixture contained 0.05 U *r*Taq polymerase (Takara, Japan), 1 µl 10× PCR buffer (1.5 µM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl), 0.8 µl dNTPs (2.5 mM), 0.33 µl (1pmol/µl) M13-tailed forward primer, 0.33 µl (10 pmol/µl) reverse primer and 0.33 µl (10 pmol/µl) labeled M13M1 primer (5'-CACGACGTTGT AAAACGAC-3') [11]. The M13M1 primer was labeled at the 5' end with NED, VIC, PET or FAM fluorescent dyes (Applied Biosystems). The PCR condition included 32 cycles of denaturing for 15 sec at 94˚C, annealing for 15 sec at 50-56˚C and extension for 30 sec at 72˚C, with post-cycling extension for 30-60 min at 72˚C on thermal cycler (Bio-Rad). Genotypes were determined as approximate allele sizes (base pairs) by the ABI 3130xl PRISM® Genetic Analyzer, and GENEMAPPER software version 3.7 (Applied Biosystems) using com-LIZ™ 500 as size standard.

2-3. Estimation of genetic parameters

In each of the three selected species (*K. bicoloratus*, *P. punctatissimus* and *P. yokohamae*), the number of alleles (Na), observed (*Ho*) and expected (*He*) heterozygosity, Hardy-Weinberg equilibrium (HWE) were examined at each microsatellite locus. Linkage disequilibrium (LD) was also computed using GENEPOP 3.4 [12]. The probability of deviations from HWE and LD between pairs of microsatellites were performed using the Markov chain method (3000 iterations).

2-4. Sequence of flanking regions of microsatellite array

Genomic DNA samples were subjected directly to PCR using the primer sets for each locus and amplification was confirmed by 1% agarose gel electrophoresis. As described in Kim et al. [8], the PCR products were purified (AMPure® PCR PURIFICATION Kit, Agencourt), and sequenced by dideoxynucleotide chain termination (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) and purification of the products of sequencing reaction (AMPure® CleanSEQ Kit, Agencourt) on an automated sequencer (ABI PRISM™ 3130). Nucleotide sequence data were analyzed by alignment of each locus for each species on the Clustal X [13]. Existence of microsatellite array was examined for the eight microsatellite DNA loci in each of three flatfish species by comparing with that of the brown sole.

3. Results and Discussion

Cross amplification was screened in 11 related species, *G. stelleri*, *H. pinetorum*, *H. dubius*, *K. bicoloratus*, *M. achne*, *P. mochigarei*, *P. punctatissimus*, *P. schrenki*, *P. yokohamae*, *T. kitaharai*, *V. moseri* in the family Pleuronectidae (Table 1). *Phz6* and *Phz12* were effectively amplified, while *Phz3* was amplified in three species. In *K. bicoloratus* and *P. yokohamae*, eight microsatellite loci were amplified by primers for brown sole and they were polymorphic. In *M. achne*, *P. punctatissimus* and *P. schrenki*, seven primer sets were effective. Five primer sets in *H. dubius* and *V. moseri*, and four primer sets in *H. pinetorum*, *P. mochigarei* and *T. kitaharai* also gave PCR products (Table 1).

Cross amplification was further examined in twenty individuals from each of three related species,

K. bicoloratus, *P. punctatissimus* and *P. yokohamae*. Number of samples (*N*), allele size ranges (R_A), number of alleles (*Na*), expected and observed heterozygosities (*He/Ho*), Hardy-Weinberg equilibrium (HWE) and existence of microsatellite array for the eight microsatellites DNA loci are shown in Table 2. In *K. bicoloratus* and *P. yokohamae*, eight microsatellite loci were amplified by primers for brown sole. Seven primer sets were effectively amplified except for *Phz7* in *P. punctatissimus*. Number of alleles per locus ranged from one to 22 in the three species and the *Phz3* locus gave the largest *Na* in *P. punctatissimus* (Table 2). No polymorphism was observed at two loci (*Phz4* and *Phz6*) in *K. bicoloratus*, one locus (*Phz12*) in *P. punctatissimus* and two loci (*Phz7* and *Phz14*) in *P. yokohamae*. *He* and *Ho* among the loci ranged from 0.66 to 0.96 and from 0.65 to 1.00 in *K. bicoloratus*, from 0.62 to 0.96 and from 0.55 to 0.95 in *P. punctatissimus*, and from 0.43 to 0.91 and from 0.40 to 0.95 in *P. yokohamae*. Significant deviations from HWE was found at the *Phz3* for *K. bicoloratus*, at the *Phz2* and *Phz3* for *P. punctatissimus* and at the *Phz3* and *Phz12* for *P. yokohamae* ($P<0.006$). Significant LD was found between the *Phz3* and *Phz6* ($P=0.005$) in *P. yokohamae*.

Cross-species amplification was possible in different flatfish species when microsatellite loci developed in brown sole were applied. More than 90% microsatellite primer sets from channel catfish are available in a closely related blue catfish [14]. All the channel catfish primers tested successfully amplify genomic DNA from flathead catfish, and 86% of the channel catfish primers successfully amplify microsatellite in white catfish [15]. We conclude microsatellite DNA markers developed from brown sole can be easily applicable for population studies, parentage assignment and mapping in other flatfish species.

Microsatellite flanking regions with more than 100 bp were compared among the three species and brown sole when good sequence data were obtained (Table 3). The results of sequencing at *Phz 3*, *4*, *8*, *12* and *14* loci showed that each locus comprised the highly homologous flanking region of microsatellite array among these four flatfish species. The mean values of homology in the other five loci were 93.7% in *K. bicoloratus*, 91.2% in *P. punctatissimus*, and 93.9% in *P. yokohamae*, when compared with brown sole. At the *Phz12*, microsatellite flanking region showed only one nucleotide transversions (T to G), when compared with homology between the brown sole and other related species examined herein. Although presence of microsatellite array was detected at three other loci, *Phz 2*, *6* and *7*, it was difficult to compare sequences of flanking regions because of highly variable sequence. Thus, we could not

conclude that these loci in other three flatfish species were homologous to those of brown sole.

Microsatellite flanking region (>100 bp) was preserved and more than 90% of nucleotides were identical among the four flatfish species (*K. bicoloratus*, *P. herzensteini*, *P. punctatissimus* and *P. yokohamae*). Similar result was reported for $(CA)_n$ repeat microsatellite in HLA-B gene of human [16]. They indicate microsatellite genotypes are not always explained by change in the number of repeat units and sometimes due to the changes in the flanking sequence. Particularly, regular transitions and transversions may offer important information among different species. Asahida et al. [17] tried to analyze phylogeny using microsatellite flanking sequences in *Sebastes* subgenera.

Consequently, we revealed that several microsatellite markers of brown sole could be used in other flatfish species. *Phz12* locus may have potential sequences to design universal primer sets for flatfish species in its microsatellite flanking regions

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Table 1. Screening of cross-species amplified microsatellite loci developed in brown sole *Pleuronectes herzensteini* in 11 different flatfish species

Pleuronectidae **Species name**	No. of samples	Microsatellite DNA markers								Number of loci amplified		
		Phz12	*Phz6*	*Phz2*	*Phz14*	*Phz8*	*Phz4*	*Phz7*	*Phz3*	++	+	-
Glyptocephalus stelleri	2	++	++	-	-	+	-	-	-	2	1	5
Hippoglossoides pinetorum	2	++	++	++	-	+	-	-	-	3	1	4
Hippoglossoides dubius	2	++	++	++	++	++	-	-	-	5	0	3
Kareius bicoloratus	2	++	++	++	++	++	++	+	+	6	2	0
Microstomus achne	2	++	++	++	++	+	+	+	-	4	3	1
Pleuronectes yokohamae	2	++	++	++	++	+	++	++	+	6	2	0
Pleuronectes schrenki	2	++	++	++	+	++	++	++	-	6	1	1
Pleuronectes punctatissimus	2	++	++	++	++	+	++	-	++	6	1	1
Pleuronectes mochigarei	1	+	+	+	+	-	-	-	-	0	4	4
Tanakius kitaharai	2	++	++	-	+	++	-	-	-	3	1	4
Verasper moseri	2	++	++	++	++	-	++	-	-	5	0	3
Alleles size (bp)		120 - 236	123 - 165	112 - 181	129 - 224	284 - 502	274 - 374	124 - 210	142 - 280			

++, amplified in two samples; +, amplified in one sample; -, no amplification

Table 2. Number of samples (*N*), allele size ranges (R_A), number of alleles (*Na*), expected and observed heterozygosities (*He/Ho*), Hardy-Weinberg equilibrium (HWE) and microsatellite region (MSR) for the eight microsatellite DNA loci cross-specific amplified in three flatfish species

Locus	*Kareius bicoloratus*						*Pleuronectes punctatissimus*						*Pleuronectes yokohamae*					
(Label)	***N***	***R_A*** (bp)	***Na***	***He/Ho***	**HWE**	**MSR**	***N***	***R_A*** (bp)	***Na***	***He/Ho***	**HWE**	**MSR**	***N***	***R_A*** (bp)	***Na***	***He/Ho***	**HWE**	**MSR**
Phz2 (NED)	20	132-234	19	0.95/1.00	0.542	Y	20	130-144	3	0.62/0.95	0.001**	Y	20	161-183	9	0.82/0.95	0.123	Y
Phz3 (VIC)	20	156-324	18	0.94/0.70	0.001**	Y	20	156-308	22	0.96/0.55	0.000**	Y	20	136-260	9	0.84/0.60	0.003**	Y
Phz4 (PET)	20	377	1	-	-	-	15	276-302	6	0.77/0.80	0.102	Y	20	279-307	13	0.91/0.70	0.034	Y
Phz6 (FAM)	20	132	1	-	-	Y	20	137-177	15	0.93/0.85	0.350	Y	20	136-142	2	0.43/0.40	1.000	Y
Phz7 (NED)	20	162-244	19	0.96/0.90	0.196	Y	-	-	-	-	-	-	20	124	1	-	-	Y
Phz8 (VIC)	20	321-341	8	0.85/0.75	0.199	Y	20	293-335	12	0.86/0.70	0.086	Y	20	285-299	5	0.78/0.75	0.677	Y
Phz12 (PET)	20	155-161	4	0.66/0.65	0.652	Y	20	150	1	-	-	Y	20	155-235	15	0.84/0.60	0.001**	Y
Phz14 (FAM)	20	190-246	11	0.89/0.90	0.806	Y	20	186-278	17	0.93/0.85	0.017	Y	20	143	1	-	-	-

**Not conformed to Hardy-Weinberg equilibrium (P<0.006, Bonferroni-corrected value); Y have a microsatellite array.

Table 3. Alignment of sequence in flanking sequence (>100bp) of cross-amplified microsatellite loci in brown sole *Pleuronectes herzensteini* and other related species *Kareius bicoloratus*, *P. punctatissimus* and *P. yokohamae*, percentage in parenthesis means homology to the brown sole sequence

Locus	Species name	Alignment of sequence in flanking region of microsatellite
Phz3	*Pleuronectes herzensteini*	AGA GAA GAG AAG AGG TGT GG- --- GTG TGT GTG TGT GTG TGT GTG TGT GTG TAT TTG CTT GTG AGC CTG TAA ATT CTG [78]
	Kareius bicoloratus	..T -.. .-- ..- --. ... CTG TG- --- --- --- ... ... TA. .G. .. ..C ... ... [78]
	P. punctatissimus	.C- -.. ... ... --. ... .TG TGT ... ... .G. G. .G. ... ... .GC T.. ..T [78]
	P. yokohamae	... ... G. ... ... -- --- --- --- --- ... ...C ... ... [78]
	Pleuronectes herzensteini	ATA GGA AGA TGT TGA TTT AAA TAA CTG ACT CTC TAA GGA TCT GAC ATA AAA ACT GAA AAA AAA GAA GAA AGC GAT AGA [156]
	Kareius bicoloratus	... ... -.. ... .- ... ... [156]
	P. punctatissimus	.G. ..G ... ... T.- ..T ... T.. ... .CT A.. ... ..G ... T.. -G. ..G ... ..- ... A.. ... ... [156]
	P. yokohamae	... ... -.. ... .- ... ... G.. [156]
	Pleuronectes herzensteini	GGA AAA A-G TGA AGA AAA AAA CCC ACC CCA GCT CTC ACA GAG TGA GTT TA [206] (100%)
	Kareius bicoloratus	... .-. ... .A. ..- --- ... ... C.. ... .. [206] (90.2%)
	P. punctatissimus	... ... .A. ..G .A. ... .-- ... C.. ..C ... ... T.. ... A.. ... .. [206] (78.7%)
	P. yokohamae	... .-. ... .A. .-- --- --- --. ..C ... ... C.C ... ..T ... .. [206] (87.8%)
Phz4	*Pleuronectes herzensteini*	TTG CTG AGG CGG GCG GAA AAG AGA GAG --- --- --- -CC C-G GCT CGG AAC GCC C-G GAC TGA TTG GAT ATT TTC AGC [78]
	P. yokohamae	... ... .G. ... ... AGA GAG AGA G.. ..A. ... TC. ... ... .C. ... ... .G. .-. ... [78]
	Pleuronectes herzensteini	AGC ACT CTG CGG CAC GAC AAG AGA TAA TGA GCT CTC TTT GAT CAG AGG AGC ATT GTT GTA TGT GAT GAT CGT CCA T-- [156]
	P. yokohamae	TT. ... ... .G. ... ...G .GG [156]
	Pleuronectes herzensteini	GTG TGT GTG TGA GTG TGT GTG TGT GTG TAC GCA GAC ATT GTG TGC ACC AGC AAC AGT GCC TGA TGA ATA T [226] (100%)
	P. yokohamae	... ...T ... ...-- --. ... A.. C.. T.. ... ... [226] (92.6%)
Phz8	*Pleuronectes herzensteini*	CTC TCA TTG GAC TGT GAA TAG TTA GTG TGT GTG CTC CAG TAA ATT AGC ATA CAC ATG GTC CTC CCT GAC AAG CAC CAG [78]
	Kareius bicoloratus	... ... .G. ... ... [78]
	P. punctatissimus	... ... [78]
	P. yokohamae	... ...C ... ... [78]
	Pleuronectes herzensteini	TCC GTG GTG GAA TGA GAG GGT GTG TGT G-- --- --- TGT CTG TGT GTG TGT GTC TGT GTG TGT GTG TGT --- --- --- [156]
	Kareius bicoloratus	... ... .GC TGT GTG ... ...G ... ... TTG TGT GTG [156]
	P. punctatissimus	... ...C .-- TGT GTG ... ... .C. ... ..G ... ...C ... ... --- --- --- [156]
	P. yokohamae	... ...-- --- --- --- --- --. T.. ... ..G ... ... --- --- --- [156]
	Pleuronectes herzensteini	--- --- --- --G TGT TTG GCT GGG AAA GGG AGA TAG TG- C-- -TG TTT GTT CAA TTT GCT TGA CTC CAG ATC AGA TGC [234]
	Kareius bicoloratus	CGT GTG TGT GT. ... ...C .TG C.. ... ... [234]
	P. punctatissimus	--- --- -GT GT. ... ... T.. ... ...C .TG C.. ... ... [234]
	P. yokohamae	--- --- --- --. ... .C. ... ...G .TG C.. ... ... [234]
	Pleuronectes herzensteini	AGG GGA CTG AAC TGT GAC CCA GGC AAT CAC AGA GAG AT [272] (100%)
	Kareius bicoloratus	... ... .T. ... ... A.. ... ... .GA .. [272] (95.5%)
	P. punctatissimus	... ... .G. ... ... A.. ... ... A.. .A [272] (95.5%)
	P. yokohamae	... T.. ... ... A.. .. [272] (96.0%)

Table 3. Continued

Locus	Species name	Alignment of sequence in flanking region of microsatellite
Phz12	*Pleuronectes herzensteini*	AAT CGA CTA GAT CAA AGA CAA GGA GCC TCT CTG GGG TGG AGA GGG ATT AAC AAC AAG TGG AAT GTG TGT GTG TAT GTG [78]
	Kareius bicoloratus	... ...- --- --- --- [78]
	P. punctatissimus	... ...- --- --- --- [78]
	P. yokohamae	... ...A ... .-- --- [78]
	Pleuronectes herzensteini	TGT GCG TGT GTG TGT GTG TGT GTG TGT GTG TGT GTA GCA GTG TGT CAG CGT TTA TCC CTC ACC TCT TCT TGG CAC AGT [156]
	Kareius bicoloratus	--- --- --- --- ... C.. ... ... G.. ... ... [156]
	P. punctatissimus	--- --- --- --- ... C.. ... .- --- --. ... ... G.. ... ... [156]
	P. yokohamae	--- --. ... ... T.. ... ... G.. ... ... [156]
	Pleuronectes herzensteini	TGT CCT TAC TGA GAC CAC CGG TGT TGA CCA GTG CTG ACC AGG C [199] (100%)
	Kareius bicoloratus	... ... [199] (99.3%)
	P. punctatissimus	... ... [199] (99.3%)
	P. yokohamae	... ... [199] (99.3%)
Phz14	*Pleuronectes herzensteini*	CCA CCT CTG CTG CCC TCT ATG ACG TAC TAG GAA TCA CTA AAG CCA CCG GTA GCT TCA GTG TGT GTG TGT GTG TGT GTG [78]
	Kareius bicoloratus	... ... G.. ... ... .T. C.. ... ... .T. .G. ... ..T ... ... [78]
	P. punctatissimus	... ...T ... .G. G.. ... ... [78]
	Pleuronectes herzensteini	TGT GCA CAA GTC AGC TCA CAC TTA ATG CTA AGT GCC TGT GTG TTT TAT AGA C [130] (100%)
	Kareius bicoloratus	... ... C.. .T. ... ... TG. ... ... .C. . [130] (89.6%)
	P. punctatissimus	... .TG --- --. CT. ... .T. ... G.. ... ... .C. . [130] (85.8%)

*Shaded regions are microsatellite array.