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Test of Mendelian segregation and linkage relationships among 69 microsatellite loci in rohu (*Labeo rohita*)

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Indian major carp, rohu (*Labeo rohita*) is an important cultivable freshwater food fish species distributed throughout Southeast Asia. It contributes nearly half of total freshwater aquaculture production (0.57 mmt) in India. To meet the ever-increasing demand of fish food, augmentation of production by developing high yielding and disease resistant breeds are imperative. This warrants use of modern technologies that can provide acceleration, reliability and sustainability to conventional breeding. The starting point may be development of genetic markers and placing them in linkage groups leading to generation of gene maps to assist in tagging of trait genes or loci. This would help in developing elite varieties of these species by employing marker assisted selections (MAS) program. Linkage maps are available for a number of fish species, including Atlantic cod (Hubert *et al*. 2010), grass carp (Xia *et al*. 2010), sea bream (Massault *et al*. 2010).

Though Indian carps contribute about 90% of total freshwater aquaculture production of India, very little genetic studies have been undertaken in these species. Only 69 microsatellite loci are so far available from rohu in literature (Das *et al*. 2005, Gheyas *et al*. 2006) and there is no linkage map so far generated in any of the Indian carp species. Linkage mapping involves measurement of recombination frequencies in the parental gametes as evidenced by co-inheritance patterns in offspring. A key consideration during map construction is selecting suitable mapping populations in order to exploit maximum polymorphism. A number of different types of pedigrees such as F1, F2 and backcross (BC), doubled haploid (DH) recombinant inbred (RI) lines are used for the purpose, depending on the requirement. Inter-specific hybrid generated from mating of closely related species is one of the strategies to increase polymorphism between parental pairs. Therefore, the objective of the present investigation was to evaluate two mapping pedigrees one intra-specific (rohu × rohu) and another interspecific rohu × kalbasu (*L. calbasu*) pedigree by studying Mendelian segregation and linkage relationship among available rohu microsatellite loci.

Rohu and kalbasu have 25 pairs of chromosomes (Zhang *et al*. 1991) and produce viable F_1 hybrids (Chaudhuri 1968). F_1 intraspecific progeny was produced (65 individuals) by crossing a wild rohu (female) with a farm rohu (male) selected for growth traits from rohu breeding programme of the Institute. F1, interspecific hybrids were generated by mating rohu females with kalbasu males. F1, hybrids and parents were screened with 10 markers prior to the spawning season to determine which parental combination would be most informative while producing BC. One backcross family (52 individuals) was produced in the spawning season by mating the one F_1 male and one rohu mother. Mapping pedigreed fish were raised in experimental ponds at Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, India and fin samples were collected for genotyping.

DNA was isolated following the conventional phenol-chloroform extraction protocol. A total of 69 rohu microsatellite loci available were PCR amplified in kalbasu. Loci that did not amplify in kalbasu were excluded from further study. PCR reactions were carried out in 20ml reaction volumes containing 1× *Taq* DNA polymerase reaction buffer, 5 pmol of each primer, 200mM dNTPs, 0.25 units of T*aq* DNA polymerase and 20 ng genomic DNA as template. Amplification was performed in a GeneAmp 9700 Thermocycler programmed for initial denaturation of 4 min at 94°C followed by 30 cycles of 45S at 94°C, 1 min at annealing temperature of corresponding primer and 2 min at 72°C, and finally a 7 min extension at 72°C. PCR products were resolved through 6% denaturing polyacrylamide gel and silver staining. Those microsatellite loci amplified in both the species were considered for PCR genotyping of

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Table 1. Rohu microsatellite loci used for linkage with accession number, repeat type, primer sequence, allele size, annealing temp. (T_a) and parental mating configuration

Locus name	Accession No	Repeat types		Primer sequences (5'–3')	Allele size	T_a	Mating configuration
Lr-01b	AJ507518	$(TG)_{14}$	F	GAC CCT TAA CCC TTG ACC TT	171	58	BC
			R	TGG GAT AAT GCA GGG AAA AC			
Lr-12[c]	AJ507524	$(CA)_{13}$	F	CAC CGC TGC TGT CCA TCA	161	58	IC
			R	AGG TCG GCC AGA TAC ACG			
Lr-14b	AJ831434	$(CA)_{12}$	F	TCA CAT GGG AAC AAC AAA CC	172	58	MIC
			R	CCG CCG CTT ACC CAT CAC			
Lr-20b	AJ831435	$(TG)_{12}$	F	CTC CAC ATG CTG AGT TAT TG	105	50	BC
			R	CTC CTT CAA GAT GCT CAA AC			
Lr-21	AJ831436	$(CA)_{11}$	F	GAT CAG AGG GTC AAT GTG G	148	58	BC
			R	CAG CAG AGT ACT ATG GAA GA			
Lr-22	AM285342	$(TG)_{19}$	F	GAT CTG TGT GTG TGT GTG C	158	58	BC
			R	GGT GGC GAC ACA ACA AAT G			
Lr-24	AJ831438	$(TG)_{17}$	F	CAA GGC GAA AAG TGT CCA T	170	56	MIC
			R	AGG AAA TTG GTA AAG TGT TTC			
Lr-26	AJ831439	$(TG)_{8}$	F	CCA GGG AGC TGC TAA GAA T	151	56	BC
			R	AGC GCT TCA TGC AGT CTA C			
Lr-27	AM231176	$(AC)_{12}$	F	TGGAAATCGAAGGCGTTTCCAC	170	60	BC
			R	AGCACTTACAGTCCATTGGCTC			
Lr-28[c]	AM231177	$(AC)_{18}$	F	TTCACGGACAGATTTGACCCAG	175	60	IC
			R	AGTCTTTTCAGGAGATTAGCAG			
Lr-32	AM231181	$(AC)_{15}$	F	ACGATTGGGCTCTCAGAAGAC	130	60	BC
			R	ACGGGTTGGAAAATGAACAGTC			
Lr-33[a]	AM269523	$(CA)_{17}$	F	AGC GAT GGA CGG GCC TAT TAC	205	61	MIC
			R	AGC CAC TGT TTA GCT TCA CAG G			
Lr-35	AM269525	$(CA)_{13}$	F	TGT GAA CAT GCA AGC TCT CAG	150	50	BC
			R	CTA GTC CCA CTC TAG TCA GCA			
Lr-36	AM269526	$(CA)_{10}$	F	AGC GTG TCT GAT GTG TGA AAG G	181	60	BC
			R	TCA GAT GCC TCC TGC ATT CTG			
Lr-37[a]	AM269527	$(CA)_{23}$	F	TGA GAT GTT CAG CAG GAG CTC	158	60	BC
			R	GAG CGT CGA GTG GCG TTT C			
Lr-38[a]	AM269528	$(GT)_{12}$	F	ATA GCA TCA CCA TCT GTT GGT G	153	59	BC
			R	TCT GCT TCA GTC ACT CAG CAC			
Lr-41	AM269531	$(GT)_{16}$	F	TCC AGT CAC CAC ATG CGT TTG	199	59	BC
			R	GTC GAT TTC ATC GTG AGG CTC			
Lr-43	AM269533	$(GT)_{20}$	F	GAT CCC AGC AGA GGC TGT G	176	57	BC
			R	AGT CTG TGC TCT CTG GAG TG			
Lr-44	AM269534	$(CA)_{25}$	F	CAC CCA GGG AGT TAG TTT CTG	232	57	BC
			R	AAA GAG CAT CAT GGC ATT GAC			
Lro-2	AM184129	$(GT)_{17}$	F	TCG ACC ATG CTT GTC TTT TGT TTA	189	61	MIC
			R	CAT GGA AGC ATC ACT TTG TTA TCG			
Lro-3	AM184130	$(GT)_{8}$	F	TTA GCC GCT TCA GTT CAT TCA	207	59	BC
			R	TTA GAT CCC CAC CGC CTT AT			
Lro-5	AM184132	$(GT)_{15}$	F	TGA CGC CGA CGT GAA TGT CAC	177	66	MIC
			R	CT GCT GAT GAC CGT CAA CAT GAC			
Lro-10	AM184134	$(GT)_{10}$	F	AGG CAG TGA GGA TAA TTG TGC TCT	114	60	BC
			R	A GTG ACC ATG TGA CAC ACG			
Lro-11	AM184135	$(GT)_{9}$	F	TTT TTCG CAA AGT TTG CAT GGC TGA A	163	68	BC
			R	CTG CCG AAAA CAC TGA ACA CGG ACA			
Lro-12	AM184136	$(CA)_{15}$	F	CAG CGC TGG ACC GAC ACC A	105	68	BC
			R	TGC TGC GGG TCA TTA GTA TTC ATC			
Lro-14[b]	AM184163	$(GT)_{14}$	F	AGG CGC AGT ATT GTT TAG GCT CTG TG	152	65	BC
			R	GC CCT CCT CAG TCA TAA AAC CCA ATC			
Lro-15	AM184137	$(GT)_{15}$	F	CGA TTG ACT GCT ATT GTA TTT TTA	164	53	BC
			R	TA GAC GAT TAT GTG GAT GGA			

(Concluded Table 1)

Locus name	Accession No	Repeat types		Primer sequences (5'–3')	Allele size	T_a	Mating configuration
Lro-17	AM184138	$(CA)_{15}$	F	GGG GCC ATT CAG ACC AGC TTA TCT AT	140	65	BC
			R	GA CCC ATT GCT GAA ATC AAC CTG AGT			
Lro-20	AM184139	$(GT)_8$	F	TCA TCT GAA CCC TTT TAT TTG	137	53	BC
			R	CGT TTA CCT GCG GAG ACA			
Lro-22[a]	AM184141	$(CA)_{15}$	F	CTA GTC CCC ATG AGT CTG TGT	157	55	BC
			R	GCG AGT GCG AGT GAG TAT GAG			
Lro-25	AM184143	$(GT)_{14}$	F	CGG TGA ATT TGC AGT GAT GTG T	184	61	BC
			R	C AAC TAC TGC AAC CTG AGA ACG			
Lro-29	AM184145	$(GT)_8$	F	AAT GGC GCT TGA ACA GAA TC	188	59	BC
			R	GCG ACC GCT TTA ATG AGA CGA			
Lro-32	AM184148	$(GT)_{17}$	F	ACC CTC TTT GTT TTG GCT CTC	136	58	MIC
			R	TCT CTT ACC CTG TTT CTC TGT			
Lro-35	AM184151	$(CA)_{11}$	F	CGC AGT GGG ATA CGC ATT ACA T	131	62	BC
			R	TCG GCC GCA GTG AGC ATC			
Lro-37	AM184153	$(CA)_9$	F	ATG TTG TGG TCA TCA TGT AAA TC	182	55	BC
			R	C AGT TTC CTC CCT TCA TAG TTT			
Lro-39	AM184154	$(GT)_{14}$	F	TCA CTG AGC ACA GGA AGG CAG GAA TG	237	69	BC
			R	ATT GGT GCC GAC GGA ACA GGA AGT CT			
Lro-42[a]	AM184157	$(GT)_{17}$	F	CCG TTC ATG CGG TGG AGG TCT	153	67	BC
			R	CTG AAG CTC AGG CTG TTC TCC			
Lro-43[b]	AM184158	$(GT)_{39}$	F	TCT CTG CGC CTG TCT ACC T	171	57	BC
			R	TGT TTA TTA AAG CAC TTT CCT CAT			
Lro-44[b]	AM184162	$(GT)_{16}$	F	TCA GTC TTT AAG CGT GTG GAG TGC	158	63	BC
			R	A TGG GAA CGA GGA GAG GAC GAA			
Lro-50	AM184161	$(GT)_9$	F	CCT AAA CGC TGC CTT GTA AAG AAG C	120	63	BC
			R	AT GCG AGG CCA CGG TGT CAG			

[a]Markers that showed deviation from normal Mendelian segregation ratio; [b]markers that were used for female mapping parent; [c]markers that were informative but heterozygous for same alleles in both mapping parents.

mapping pedigrees. 10 offspring from each family along with the respective parents were PCR genotyped to evaluate both the population. Loci that were heterozygous for same alleles in both the parents were excluded from the analysis. Genotype configurations of markers were categorized into three segregation types when null-allele segregation was allowed: 1:1:1:1-ratio type (female × male: AB × CD or AB × AC), 1:1 female type (AB × AA or CC), and 1:1 male type (AA or CC × AB).

All the statistical analyses were done using LINKMFEX version 2.3 (http://www.uoguelph.ca/~rdanzman/Software/LINKMFEX), which handles the data containing various genotype configurations. Segregation data from expected 1:1:1:1-type markers into 1:1 female and 1:1 male type was partitioned by creating maternal and paternal datasets. This is done by converting genotypes from 1:1:1:1-ratio type (female × male: AB × CD or AB × AC) to 1:1 female type (AB × AA or CC), and 1:1 male type (AA or CC × AB). All the informative loci were tested for departure from expected Mendelian segregation ratios using Chi square test for goodness of fit ($\alpha = 0.05$) with their respective degrees of freedom. Loci that deviated significantly from the Mendelian segregation ratio were excluded from the analysis. Linkage analysis was conducted through a series of pair wise comparisons between loci using LINKMFEX. Pair-wise recombination estimates obtained with module LINKMFEX were used as input into module MAPORD to determine linear assignments of markers within a linkage group (LG). Markers were linearly aligned in each linkage group, converting the recombination rates into the Kosambi's map distance (centi-Morgans) with module MAPDIS and map of LGs was drawn by MapDraw program (Liu and Meng 2003). A minimum LOD score of 3.0 was interpreted as significant linkage (Botstein *et al*. 1980).

The purpose of this study was to evaluate 2 mapping pedigrees using available rohu microsatellites for establishing linkage relationship among markers. An important step in designing mapping studies is to find markers that show polymorphism. In the preliminary screening, rohu F_1 panel showed very low polymorphism (12%) while interspecific panel exhibited 65% loci to be informative for linkage analysis. Very few informative loci exhibited by intraspecific panel here could be due to less genetic distance between the wild and farm rohu as compared to genetic variation of 2

different species (interspecific). Out of 69 rohu microsatellite loci studied, 61 could be cross amplified in kalbasu. 40 loci (Table 1) were informative in interspecific mapping population from which 5 loci deviated from normal Mendelian segregation ratio at (α = 0.05) and 2 loci were heterozygous for the same alleles in both parents.

The remaining 33 loci (25 in male, 3 in female and 5 loci in both parents) were informative. Eight loci showed linkage association with a minimum LOD score of 3.0 forming 3 linkage groups in male parent spanning 67.29 cM of the genome and none was found to be linked in female.

Sixty-one loci from a total of 69 (88%) cross amplified in kalbasu genome, which is comparable with our previous report (Das *et al*. 2005). For a given locus, parents of outbred population can be in backcross (BC), multiple backcross with 3 alleles (MBC), intercross (IC) and multiple intercross for 3 or 4 alleles (MIC) (Williams 1998). The segregation ratios are 1:1 for backcross and multiple backcross, 1:2:1 for intercross and 1:1:1:1 for multiple intercross configurations. More than 80% (33 loci) of the informative markers exhibited BC mating type configuration (Table 1). This skewed observation towards BC type mating configuration is likely due to locus homozygosity, but each species is homozygous for different alleles. Thus an interspecific F_1 would have more heterozygous loci, and one would expect an F_1 mated with rohu to have the BC type of segregation. Five loci (12.5%) were found distorted, which is comparatively higher than that of turbot (6.8%) (Ruan *et al*. 2010). However, this is similar to spotted halibut (14.4%) (Ma *et al*. 2010). Several reasons may account for the observed marker distortion, including competition among gametes for preferential fertilization (Lyttle *et al*. 1991) and sampling in finite mapping populations or markers types. As far as linkage is concerned, 8 loci could be assigned to 3 LGs of male hybrid parent covering only 26.6% of the informative markers under significant linkage. One reason could be due to less number of loci (33 markers) finally available for linkage analysis after elimination of non informative and distorted markers. Given the haploid chromosomes number (n, 25), informative markers found for linkage analysis in this study may be below optimum to place most of the loci in LGs. However, the unlinked loci would find place in LGs with increase in the number of informative markers. The loci that were optimized but not informative in this pedigree may be useful in other mapping families. The genomic resource generated for the carps during the study is first of its kind in Indian carps and would help generating a first generation linkage map of carp.

SUMMARY

Two mapping pedigrees (1 intraspecific and 1 interspecific) were evaluated with 69 rohu microsatellite loci. Deviations from the expected Mendelian segregation ratios were tested and linkage relationship among the loci established. Interspecific mapping pedigree (rohu×kalbasu) was 5 times more informative than rohu F_1 pedigree. 61loci got cross amplified in kalbasu with 40 markers informative in interspecific mapping pedigree. Altogether, 5 loci deviated from normal Mendelian segregation ratio (α = 0.05) and 8 loci could be assigned to 3 linkage groups at LOD > 3.0. The microsatellite linkage groups and the mapping families generated in this study represent the beginning of a genetic map for Indian major carps.

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