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Preliminary genetic linkage map of Indian major carp, *Labeo rohita* (Hamilton 1822) based on microsatellite markers

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Abstract

Linkage map with wide marker coverage is an essential resource for genetic improvement study for any species. Sex-averaged genetic linkage map of *Labeo rohita*, popularly known as "rohu" widely cultured in Indian sub continent was developed by placing 68 microsatellite markers generated by a simplified method. The parents and their F1 progeny (92 individuals) were used as segregating populations. The genetic linkage map spans a sex averaged total length of 1462.2 cM, in 25 linkage groups. The genome length of rohu was estimated to be 3087.9cM. This genetic linkage map may facilitate systematic searches of the genome to identify genes associated with commercially important characters and marker-assisted selection programs of this species.

Key Words: *Labeo rohita*; Microsatellite; consensus linkage map; Indian major carp

Running Title: Genetic Linkage map in *Labeo rohita*

Introduction

Labeo rohita commonly known as "rohu" is one of the Indian major carps used successfully in commercial aquaculture in Indian sub continent at least for the last six decades. Integration of modern molecular biological tools along with conventional breeding programs provides speed, reliability and sustainability in producing improved varieties. An ongoing selective breeding program in rohu which started in1992 at this Institute has achieved 17% higher growth per generation (Mahapatra *et al*. 2007) where as markers needed to achieve further accelerated genetic improvement in rohu have just begun (Patel *et al*. 2009; Sahu *et al*. 2013; Robinson *et al*. 2012). The outcome of such an effort is the generation of a genetic linkage map based on SNP derived from transcribed sequences. (Robinson *et al*. 2014).

Genetic linkage map for a number of aquatic species have been constructed (Moen *et al*. 2008; Jiang *et al*. 2013; Liu *et al*. 2013, Hollenbeck *et al*. 2015; Hermida *et al*. 2014; Keong *et al*. 2014; Dor *et al*. 2014) using different types of polymorphic markers. Owing to their abundance in the genome, high polymorphism, ease in analysis using PCR, etc. microsatellite markers, are widely utilized for construction of linkage map of many aquatic species though it is expansive and time consuming to develop such markers. The advent of next generation sequencing has allowed fast and cost effective development production of microsatellite markers (Perry and Rowe 2011). In this study, we report: 1) the development of microsatellite markers, 2) the use of these markers in the construction of a genetic linkage map of rohu and 3) the comparison of the map generated by Robinson *et al*. (2014) using SNPs in the same species.

Materials and methods

SSR identification and marker development

SSR markers were developed as described by Dubut *et al*. 2010. Genomic DNA of *L. rohita* was isolated from fresh fin tissue samples using standard phenol-chloroform method (Sambrook and Russell 2001). *Rsa*I (New England Biosystems, Ipswitch, Massachusetts, USA) digested DNA fragments ligated to standard oligonucleotide adapters were hybridized with biotin-labelled CT and AGG probes. The SSR enriched libraries were subjected to sequencing using 454 GS FLX Titanium pyro-sequencing platform, and high quality reads having more than 200 bp were taken

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for further processing. QDD program to find microsatellite sequences (a minimum of six repeat units for Di- and Tri-nucleotide, five for Penta- and Hexa-nucleotides and four for Hepta-nucleotides, considered as microsatellite unit) and Primer 3 program (Meglécz *et al*. 2010) was used to design primers for the microsatellite. Simultaneously, another SSR enriched (CA) library prepared in pGEMT Easy vector (Promega, Madison WI, USA) was subjected to Sanger sequencing using Big Dye terminator version 1.1cycle sequencing kit in an ABI 3770 sequencer (Foster City, USA). Sequences obtained were manually edited and primers were designed from unique microsatellite containing sequences using Batchprimer3 (You 2008).

Mapping population

Single F1 full-sib family as linkage mapping panel was obtained by crossing a sire selected for body growth (5th generation) with an unselected dam in the year 2008. Fin clips after collection from the parents and the offspring (yearling) were stored in 95% ethanol.

DNA isolation and polymorphism analysis

Genomic DNA was isolated from fin tissues by phenol and chloroform method. DNA quantity and quality were checked by spectrophotometry and 0.8% agarose gel respectively. The source of the microsatellite markers used in this study was as follows: 493 loci were developed in the present study, 47 loci were previously developed in our laboratory (Patel *et al*. 2009; Das *et al*. 2005) and the rest (135) were from the public database. The markers heterozygous at least in one parent were considered informative and used for genotyping 92 progeny and their parents. Primers were procured from Applied Biosystems (Foster City, USA) with forward primer labelled with FAM, PET or NED fluorescence dye. Polymerase chain reactions were carried out in 15 µl volumes containing1X Taq polymerase reaction buffer with 1.5 mM $MgCl_2$, 200µM dNTPs, 5 pmol of both forward and unlabelled reverse primers, 0.25U of Taq DNA polymerase (Bangalore Genei, India) and 20ng of genomic DNA. The PCR conditions were optimized for each multiplex. The thermal cycles consisted of initial denaturation of 4 min at 94°C followed by 30cycles of 45 s at 94°C, 1 min at the annealing temperature of the corresponding primer (Supplementary Table 1) and 2 min at 72°C and finally an extension of 15 min at 72°C. One micro liter PCR products and 0.1 µl GeneScan™–500 LIZ™ size standard (ABI) were added to 9.9 µl of Hi Di™ formamide. The mixture was subjected to genotyping on an ABI 3770

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sequencer (Applied Biosystems), and genemapper v. 3.7 software (Applied Biosystems) was used to edit and analyze the data.

Microsatellite segregation and Linkage analysis

Genotype data of parents and 92 progeny at 140 microsatellite loci was subjected to Goodness of fit of the observed-to-expected allelic ratios using the Chi square test. Markers heterozygous in at least one of the parents were defined as polymorphic. Markers segregating in Mendelian ratios were used for the construction of map. Genotype configuration of markers was categorized into four expected segregation types: 1:1:1:1 ratio type (♀ X ♂: AB X CD or AB X AC), 1:2:1 type (AB X AB), 1:1 ♀ type (AB X AA or CC), and 1:1 ♂ type. Genotype data of 140 microsatellite markers were then analysed to construct genetic linkage map of rohu using Joinmap Ver 4.1 (Kyazma, Wageningen, Netherland) with cross pollinating (CP) coding scheme, which handles F1 out bred population data containing genotype configurations with phase unknown. Linkage between markers was examined by estimating LOD scores for recombination rate (θ) on the basis of maximum likelihood method with the EM algorithm. The Kosambi mapping function was used to convert recombination units into genetic distances. The mapping analysis was conducted by using a minimum LOD score of 3.0. The linkage map was visualized using Map Chart version 2.2 (Voorrips 2002). On the basis of consensus map, expected genome length was obtained by the following two methods. First) the average spacing between markers, which is calculated by dividing the total observed map length by the number of marker intervals, was estimated, followed by adding twice the "s" value to the observed map length of each linkage group (EG1, Fishman *et al*. 2001). Second) the observed map length of each linkage group was multiplied by (m+1) / (m-1), where m is the number of markers that were placed at different positions on the linkage group (EG2, Chakravarti *et al.* 1991). The average of the EG1 and EG2 was taken as estimated genome length (Ge) of rohu. The observed genome length (Go) was calculated as the total length of the framework map. The map coverage was determined by Go/ Ge. Comparison of meiotic recombination rate among parents was performed by taking common maker pairs.

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Results

SSR marker development

Next generation sequencing of enriched SSR library using Roche 454-GS-FLX sequencing platform generated 6967 contigs. Of these, 3677 (52.7%) contigs contained di-, tri- and tetra nucleotide repeat motifs. Only 387 (5.5%) microsatellite containing sequences (GenBank accession number JQ862039 to JQ862292) could be used for primer designing. A total of 255 (3.6%) primers were designed to get PCR product size ranging from 98 bp to 350 bp. In addition to next generation sequencing mentioned above, 1840 clones from enriched second SSR library were subjected to Sanger sequencing, which resulted, 748 (40.6%) di-, tri- and tetra nucleotide repeat motifs within the sequence reads. Of which, primers could be designed from 238 unique sequences (12.9%, GenBank Accession number JN581132 to JN581366). It is interesting to note that the enriched SSR library when subjected to Sanger's sequencing method provided more number of microsatellite markers in our hand though next generation sequencing technology is economical, time saving and less labour intensive.

Linkage analysis

Before embarking to linkage analysis both parents of the reference family were screened for polymorphism at all the microsatellite loci. The marker when heterozygous in one of the parents was taken as informative and used for genotyping the F1 progeny along with their parents. Of the 675 microsatellite loci initially screened, only 155 loci were informative for two point linkage analysis. The genotyping data on these loci were inspected manually. Of the 155 loci, 10 were monomorphic and 5 resulted ambiguous band pattern and therefore, segregation data on 140 markers was used for linkage analysis. Segregation patterns of all the loci were as follows: 42 with AB X CD (12 markers) or AB X AC (30 markers), 36 with AB X AA or CC and 49 with AA X AB. A total of 17 loci had segregation distortion and were discarded. The remaining 123 SSR markers generated robust and easily interpretable genotypes that could suitably be used for genetic mapping. A total of 68 loci were included in the framework map across 25 linkage groups (LG), 5 of which contained four markers, 8 of which contained three markers and 12 LGs had two markers. The consensus framework map consisted of 25 linkage groups (Fig. 1) spanning a total length of 1462.2 cM. The size of the linkage group ranged from 5.1 to 164.6 cM, and the number of markers in the linkage group varied from 2 to 4. The average marker spacing

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was 34cM. LG1 had largest average interval of 41.15 cM and LG25 had smallest average interval of 2.35 cM. The average estimated genome length was 3087.9cM for the consensus framework map of *Labeo rohita* and the genome coverage of the present framework map was 47.6%.

Differences in recombination between sexes

The availability of common microsatellite markers allowed a comparative evaluation of meiotic recombination rate in the male and female. A significantly higher recombination rate was observed in the female map (545.9cM0 in comparison to male map (348.8cM). Adding the length of the interval between the loci, it rendered a total length of 96.7cM and 162.2cM in the male and female map, respectively. Thus the recombination rate in the female was 1.67 times higher than in male.

Discussion

Genetic maps provide important genomic information and allow the exploration of QTL, which can be used to maximize the selection efficiency of target traits. Linkage analysis and map construction using molecular markers is more complex in full-sib families of out-breeding species than in progenies derived from homozygous parents. For example, markers may vary in the number of segregating alleles, one or both parents may be heterozygous, markers may be dominant or co-dominant, and usually the linkage phases of marker pairs are unknown. In this present study, we constructed a microsatellite based first generation consensus genetic linkage map using unambiguous genotyping data for 68 markers in rohu. The consensus linkage map has 25 linkage groups, which agrees with the diploid chromosome number of 2*n*=50 and is in confirmation with the linkage map data provided by Robinson *et al*. (2014) for the same species using SNP markers.

SSR marker development

Next generation sequencing has been successfully used for rapid development of genetic markers including SSRs (Davey *et al*. 2011; Santana *et al* 2009; Martin *et al*. 2010). Massive parallel sequencing in recent times has been employed for the development of SSR markers in many organisms to reduce the cost, labour and time required. (Kircher and Kelso 2010; Abdelkrim *et al*. 2009; Yu *et al*. 2011; Csencsics *et al*. 2010). Various microsatellite development protocols based on partial genomic library enriched for microsatellite are available in the literature (Liao *et*

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al. 2007; Pardo *et al*. 2007; Abdelkrim *et al*. 2009; Wang *et al*. 2010). However, marker development following Sanger sequencing has been shown to be more effective (Malausa *et al*. 2011). In the present study, both Sanger sequencing as well as 454 GS FLX Titanium pyrosequencing was employed to develop microsatellite markers. Next generation sequencing resulted in only 3.6% sequences suitable for primer designing compared to 12.9% in conventional Sanger DNA sequencing. This might be due to the shorter and less accurate reads obtained from pyrosequencing in the present investigation. In the honey bee (*Apis melira*) and zebrafish (*Danio rerio*), only 1.2% to 5.4% sequences could be used to design primers for microsatellite marker development by 454 FLX titanium pyrosequencing.(Malausa *et al*. 2011; Sarinen and Austin 2010).

Linkage analysis

Like many other aquaculture species, mapping resources such as purebred lines, near isogenic lines and inbred lines are not available in *Labeo rohita.* Pseudo-test cross strategy for linkage mapping has been successfully employed in various fish species (Liu *et al*. 2003; Coimbra *et al*. 2003; Ohara *et al*. 2005; Wang *et al*. 2007) for quick mapping and QTL analysis. The F1 pseudo-testcross mapping strategy used for genetic linkage map of highly heterozygous organisms was employed for the construction of the present microsatellite based rohu linkage map. Map built on microsatellite markers may facilitate transfer to other families or strains allowing further addition of markers on a genome wide anchored map, which may not be possible in other markers like AFLP (Li *et al*. 2012). This study shows that microsatellites are effective markers for linkage mapping in *Labeo rohita* as has been shown for other fish and shellfish species (Sekino and Hara 2007; Shen *et al*. 2007; Nomura *et al*. 2011; Liu *et al*. 2012). The proportion of informative loci observed in the present study was 20.7%, which is lower than other aquatic species (Zhu *et al.* 2006; Ning *et al.* 2007). This is probably due to the fact that without prior polymorphism assessment, all the SSRs were randomly screened as markers in the reference family. SSR markers informative in both parents were tested against 1:1:1:1 (df = 3) and 1:2:1 (df = 2) segregation ratio, and informative in one parent against 1:1 (df = 1) (Zhang *et al*. 2006). Distorted segregation among DNA markers varies according to species and the nature of the mapping populations. Lower level of segregation distortion (12.14%) in SSR markers were

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observed in the present study compared to other studies e.g. 16% in catfish (Liu *et al*. 2003), 13.3% in rainbow trout (Young *et al*. 1998), 21% in guppy (Shen *et al*. 2007), 27% in Pacific oyster (Li and Guo 2004) and 30.45% in Zhinkong scallop (Zhan *et al*. 2009). Segregation distortion is a frequently encountered problem in mapping populations (Jiang *et al*. 2000) and several factors such as presence of genomic structure difference between parents of mapping populations (Truco *et al*. 2007; Hwang *et al*. 2009), errors in marker genotyping, amplification of the same size fragment from several different genomic regions (Faris *et al*. 1998), distortion of the transmission between genetically divergent genome (Fishman *et al*. 2001), sampling in finite mapping populations and preferential fertilization and zygotic selection (Rick 1969) are responsible for this phenomenon.

This is the first report on microsatellite based genetic linkage map in Indian major carp, *Labeo rohita* constructed using an F1 full-sib family reference population. The percentage of markers placed in LGs with significant linkage (LOD $\geq$ 3.0) in the present investigation is comparatively less than reported in other aquatic species (Sekino and Hara 2007; Shen *et al*. 2007; Bouza *et al*. 2007; Li *et al*. 2012; Liu *et al.* 2013). This may be due to several factors like marker polymorphism, distribution of markers in the genome, cross- over distribution in the genome, mapping population size and type, and mapping strategy (Liu 1998).

The resulting consensus linkage map spans a total sex-averaged length of 1462.2 cM with 25 LGs, with an average 2.72 microsatellites per LG ranging in length from 4.7 cM to 164.6 cM with an average spacing of 34 cM. Sex specific linkage maps were reported in rohu based on SNP markers. The length of genetic map, average spacing and maximum interval for female and male maps were 1384 cM and 1393.5 cM, 1.32 cM and 1.35 cM and 12.7 and 37.1 cM, respectively (Robinson *et al*.2014). In the current microsatellite based linkage map, number of markers placed in each LG is much less than the SNP based linkage map reported earlier (Robinson *et al*. 2014). Basing on the current linkage map the average genome length of rohu was estimated to be 3087.9cM which is much higher than the length estimated by Robinson *et al*. (2014). This might be due to poor marker coverage in the present study. The genome size of the consensus map appears to be short as evident from estimated genome length, because of the

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limited number of SSR mapped. Thus the current map provides only 47.6% coverage of the rohu genome in contrast to 99% coverage reported by Robinson *et al*. (2014). Further the genome coverage in the present study is less when compared to other teleost linkage maps like channel catfish (Liu *et al*. 2003), rainbow trout (Guyomard *et al*. 2006), turbot (Bouza *et al*. 2007), silver carp and bighead carp (Liao *et al*. 2007), yellow croacker (Ning *et al*. 2007), Atlantic salmon (Moen *et al*. 2008) and Atlantic cord (Hubert *et al*. 2010).

Differences in recombination between sexes

In most of the organisms differences in recombination rate is seen between sexes (Barendse *et al*. 1994; Ellegren *et al*. 1994; Dib *et al.* 1996; Sakamoto *et al*. 2000; Waldbieser *et al*. 2001; Singer *et al*. 2002). Higher recombination rate near the centromere in the female and near the telomere in the male were observed in rainbow trout and zebra fish (Knapik *et al*. 1998; Sakamoto *et al*. 2000). In the present investigation sex specific difference in recombination rate for specific pair of linked markers was observed. Similar results were also reported in rohu by Robinson *et al*. (2014). Higher recombination rate was shown in the female map when common informative markers were taken into account (Fig.2). Adding of the map distances for common maker pairs resulted in 96.7 and 162.2 cM in the male and female map, respectively. Therefore the ratio of female: male recombination rates for shared markers were 1.67:1. Though molecular mechanism responsible for differences in recombination rates between the two sexes is not well understood, higher recombination rate in the female has been observed in several teleost species. For instances, the female: male rates are 8.26:1 in the Atlantic salmon (Johnson *et al*. 1987; Moen *et al*. 2004; Moen *et al*. 2008) 3.25:1 in rainbow trout (Sakamoto *et al*. 2000), 1.6:1 in catfish (Kucuktas *et al*. 2009) and 1.48:1 in the European sea bass (Chistiakov *et al*. 2005). Our result with recombination rate of 1.67 (female):1(male) follows the general pattern found in other teleost fish. Microsatellite based linkage map in rohu developed, which consisted of 25 linkage groups covering a total length of 1462.2 cM with an average interval of 34 cM. The present map based on microsatellite markers is highly complementary to the existing map and would be essential for saturating rohu genetic map.

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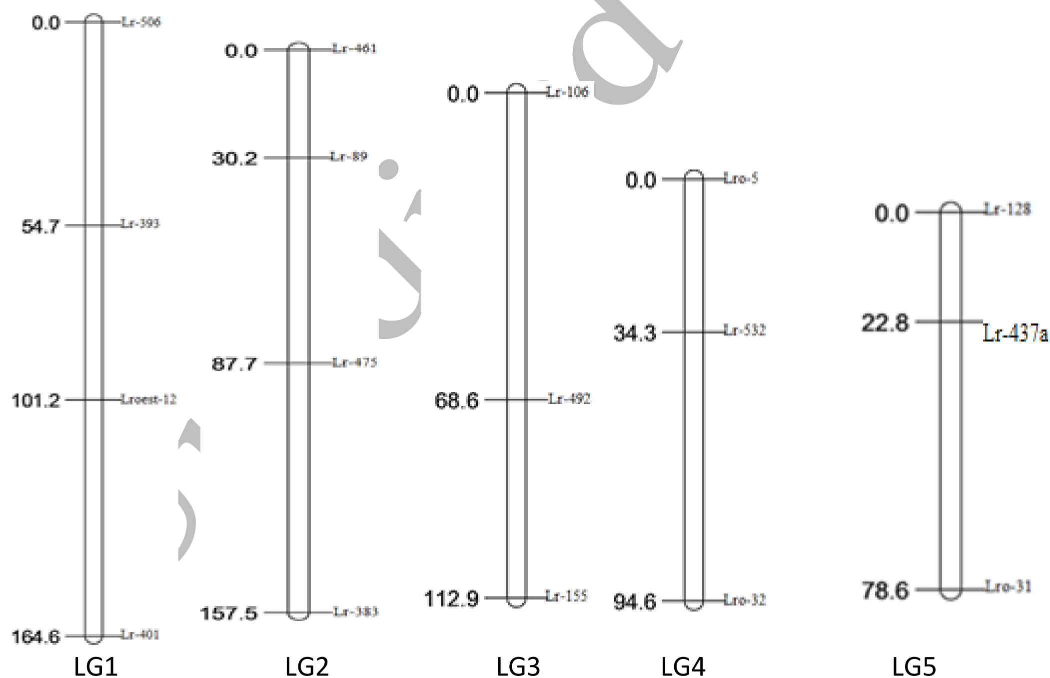
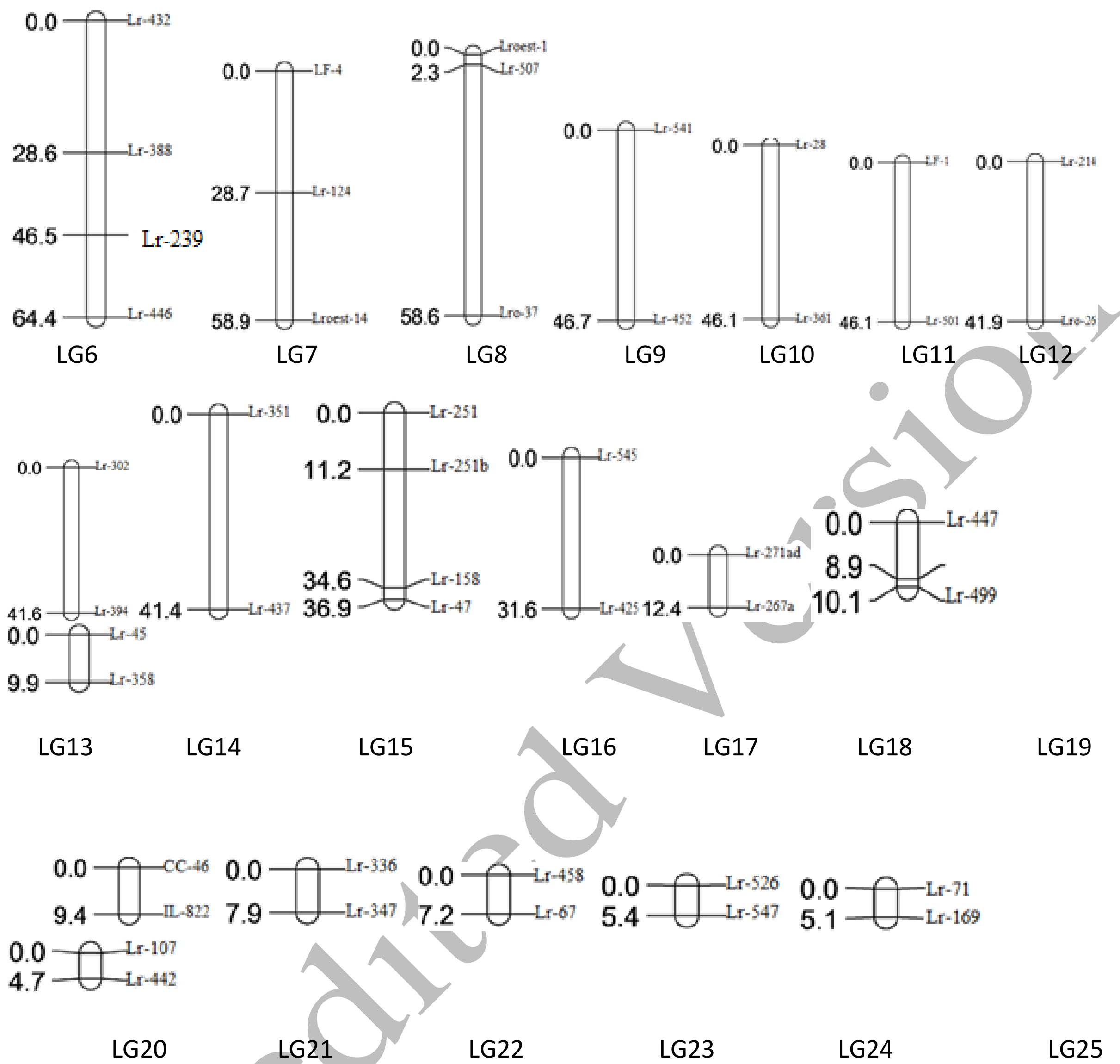
Figure legends

Fig. 1 Microsatellite based consensus genetic linkage map of rohu using F1 full-sib family.





Distances in centiMorgan, (cM) Kosambi are indicated on the left side. Acronym of the markers on the right side.

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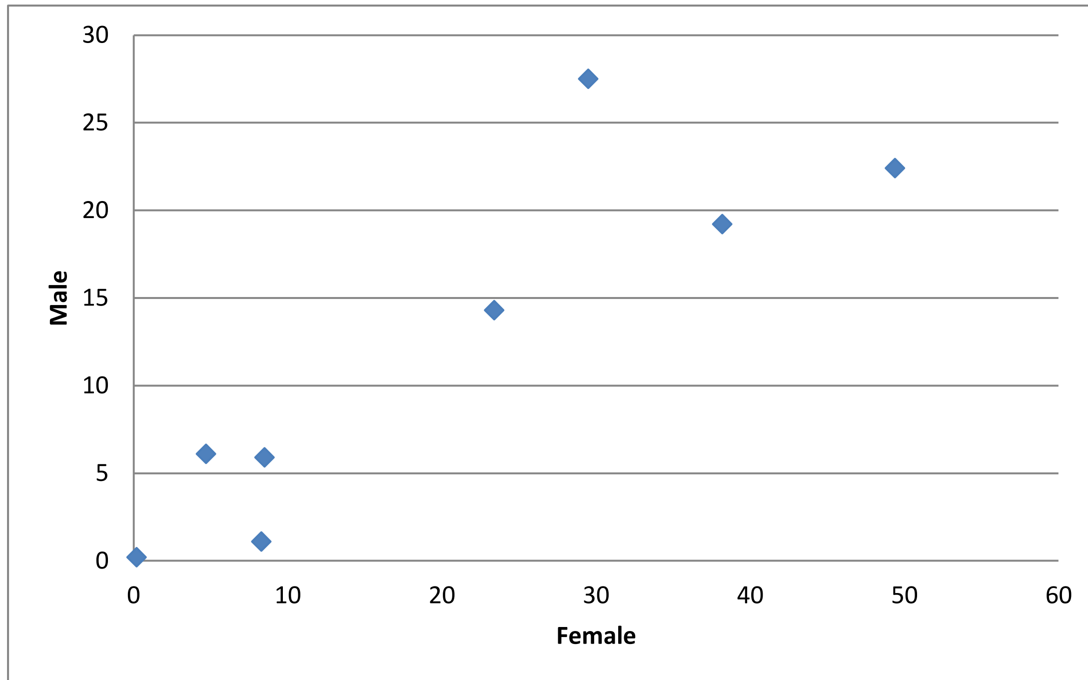
Fig. 2 Differences in recombination frequency in male and female parents



Recombination distances are in cM. recombination distances in Male are in Y-axis and in Female in X-axis.

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Supplementary Data

Table 1. Summary of characteristics of the microsatellite mapping panel in *Labeo rohita*

Serial no.	Microsatellite	GenBank Accession	Primers (5□------------------------3□)	Repeat motif	Tm	Product size
1	Lro-12	AM184136	F: CAG CGC TGG ACC GAC ACC A	(CA)15	58	105
			R: TGC TGC GGG TCA TTA GTA TTC ATC			
2	Lr-28	AM231177	F: TTCACGGACAGATTTGACCCAG	(AC)18	56	175
			R: AGTCTTTTCAGGAGATTAGCAG			
3	Lr-37	AM269527	F: TGA GAT GTT CAG CAG GAG CTC	(CA)23	57	158
			R: GAG CGT CGA GTG GCG TTT C			
4	Lr-43	AM269533	F: GAT CCC AGC AGA GGC TGT G	(GT)20	59	176
			R: AGT CTG TGC TCT CTG GAG TG			
5	Lr-45	AM269535	F: TGC ACA CCT CGA GGT TAG CAA C	(CA)15	57	256
			R: TCA GCT CCA CAT TTC ACC ATG			
6	Lro-2	AJ507519	F: TCG ACC ATG CTT GTC TTT TGT TTA	(TG)11	56	189
			R: CAT GGA AGC ATC ACT TTG TTA TCG			
7	Lro-25	AM184143	F: CGG TGA ATT TGC AGT GAT GTG T	(TG)14	56	184
			R: C AAC TAC TGC AAC CTG AGA ACG			
8	Lro-26	AM184144	F: AGA TCA TTG CTG GGG AGT GTT TAT	(GT)17	58	217
			R: GAC CTG CCT GTG CCA TCT GTA			
9	Lro-31	AM184147	F: CAT AAT AGC AGT GGC GAG CAG	(GT)21	56	188
			R: TTA GAT CCC CAC CGC CTT AT			

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10	Lro-32	AM184148	F: ACC CTC TTT GTT TTG GCT CTC	(GT)17	58	136
			R: TCT CTT ACC CTG TTT CTC TGT			
11	Lro-37	AM184153	F: ATG TTG TGG TCA TCA TGT AAA TC	(CA)9	56	182
			R: C AGT TTC CTC CCT TCA TAG TTT			
12	Lro-39	AM184154	F: TCA CTG AGC ACA GGA AGG CAG GAA TG	(GT)14	57	237
			R: ATTGGTGCCGACGGAACAGGAAGTCT			
13	Lro-43	AM184158	F: TCT CTG CGC CTG TCT ACC T	(AG)24	59	171
			R: TGT TTA TTA AAG CAC TTT CCT CAT			
14	Lro-44	AM184162	F: TCA GTC TTT AAG CGT GTG GAG TGC	(GT)16	58	158
			R: A TGG GAA CGA GGA GAG GAC GAA			
15	Lro-5	AM184132	F: TGA CGC CGA CGT GAA TGT CAC	(TG)20	56	177
			R: CT GCT GAT GAC CGT CAA CAT GAC			
16	Lroest-12	GR958186	F: GATACCAGACTGCCTGCCTATT	(GAT)5	57	199
			R: GCAAACAACTTCAGGCTCTTCT			
17	LROEST-14	GR958112	F: ATCATCTCAGGGTTTCTGTTGG	(ATT)4	57	192
			R: ACTGTGTGGTAGCGGTTACCTT			
18	LROEST1	GR426891	F: CACCACAGCACTTAACACTTAC	(AAGA)8	57	152
			R: CTAGAGTGATCCTCAGGGATT			
19	Lr-47	JN581132	F: CCCAACTCCTAAGAAGACCAGA	(AC)43	56	248
			R: GTGTAGGGTAGGTGTAGGGTGA			
20	Lr-67	JN581335	F: CAGCAGGACTGAAGAAATGTATG	(AC)19	56	289

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			R: CCAATAAATGTTGACCTTGTGG			
21	Lr-71	JN581156	F: CGAATCATTCACACGTGCTT	(CA)36	57	193
			R: TCTCCAGAGGAACCCATAGC			
22	Lr-89	JN581172	F: CGTGAACACGCGAAACAG	(AC)34	58	141
			R: CGACTCATGACATGTGCTTGTT			
23	Lr-99	JN581181	F: ACGTTCCTGACAAAACAGAGGT	(AC)44	58	193
			R: CGTACACATCCCTGACCAATC			
24	Lr-106	JN581188	F: AGTCGGATTCAAAACGAAACAG	(GT)7	56	182
			R: TGATAAAGTGTCGTAGAGGAATGG			
25	Lr-107	JN581189	F: CCAAATATGTTTTGAGGCCATT	(AC)10	57	191
			R: TGAAGGCCAAAGCTCTGTAA			
26	Lr-120	JN581201	F: ACTCCTTCAGTGTGGCAGTGT	(CA)6	59	215
			R: GTGTTTTCTCCTCAGGTGTCAG			
27	Lr-124	JN581204	F: CAGTCAATCCAGCGTCTCCT	(TGTC)5	57	196
			R: GCCACTTTACCACTTTTGCTGT			
28	Lr-126b	JN581206	F: ACAAATGTCCCACAAGCTCAAC	(CA)21	58	203
			R: GACAGGCTTTATTCTTCCTCCA			
29	Lr-128	JN581208	F: AGAGCCCATCTCTCCTGTCTTT	(CA)32	56	193
			R: GAGGAGGGGTCAATATGTGTGT			
30	Lr-131	JN581361	F: TGGTCCCATAACGTGATGAAT	(CA)9	59	168
			R: ATTTGCTTAGAAGCGCCAAC			
31	Lr-148	JN581227	F: AACACCCTCATTGTCTGATGAA	(CA)28	56	222

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			R: TTTAGGTGTAGGGTAGGTGTAGGG			
32	Lr-155	JN581232	F: AAGCCAAACGACTAAACTCACA	(AC)28	57	202
			R: GGAACAGTGTGTGCTGGACTAA			
33	Lr-158	JN581271	F: CAGCAAACACACTCCTGTCAAA	(AC)15	58	274
			R: GACGCGCAAATCAAAGTGAG			
34	Lr-162	JN581239	F: GCGAAACAGCAGCAACACT	(AC)34	58	209
			R: AAGAGAAGGCTTCACCTGGA			
35	Lr-169	JN581244	F: AAGCTCAGGCTGTCCATCTC	(CA)16	58	194
			R: GAGCAGACCACCTCCAACTC			
36	Lr-185	JN581353	F: GATCTCTGTGTGCAAGTAATG	(TG)18	58	182
			R: GGTCCCGAAATTCTCTCTGG			
37	Lr-189	JN581257	F: GATCAGAGCAATATTGGGGTTT	(CA)13	56	219
			A: AGAGCTGCTGTGTGCAGAA			
38	Lr-190a	JN581258	F: AGAGACTCGCTCCCTGGACT	(GT)11	55	168
			R: ATTCTGCATCCCAAACAGGA			
39	Lr-191	JN581259	F: ATCTCATGCCATTGTGCAGT	(CT)7	56	179
			R: CGCACGTGTCACCTTTCTAA			
40	Lr-192	JN581260	F: CCAAACTATTGAACGGCAGTG	(AC)19	58	104
			R: TATGGAGAGTCCCCACAAGG			
41	Lr-206	JN581273	F: GAAGTGTTTGTGTGGCTGGA	(TG)25	59	182
			R: CCGGTGGGATCTGTGTATG			
42	Lr-209	JN581276	F: CAATGACCATGCTAAGCACTTC	(AC)20	55	181

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			R: TGTTCATTGATATGCGTAACAGC			
43	Lr-214	JN581279	F: ATGTGATTCATATAGGGCAGGTC	(GT)16	59	100
			R: GATCACACACGATTACCATCC			
44	Lr-225	JN581289	F: CCCAGACTACATTTCCCATCA	(CA)6	56	190
			R: CAGGCAGGTAATCAGAACAAGA			
45	Lr-226	JN581290	F: TGCTGTCCTCGTAAACCAAA	(GT)6	55	194
			R: GCTTGACGCAACACAGACAC			
46	Lr-227	JN581291	F: GGCTGAGATTTGCGTGTTCT	(AC)12	55	214
			R: GGGCGATAGAAAATAGGGTTTG			
47	Lr-228	JN581292	F: TCCTCTGAAGAAAGCAAGAGAGA	(CA)18	54	200
			R: AATGGCACTGAGGTAGGACACT			
48	Lr-229	JN581293	F: TTATGTGGTCATTAACGGTGGA	(GT)11	59	147
			R: ACTGGACCAACCAGGGATTAC			
49	Lr-239	JN581303	F: ATATCGAGGTGTCCGATGATG	(AC)19	59	225
			R: GGGTTAGGGTAAGGGGATAGAA			
50	Lr-251a	JN581315	F: GAGGTCAGTTGGTCAGAGTTCA	(CA)15	58	193
			R: ACCCTTTCACACCCCTCTTATT			
51	Lr-251b	JN581315	F: CACCTGCTCCCTACAATTTGAC	(AC)6	58	205
			R: ACAGTCCTTTCCATTGCCTTT			
52	Lr-267a	JN581331	F: CAGTCTGACCATCCTGCCTTT	(AC)29	58	203
			R: GGACTAGGTTTAGGTGTAGGGTGA			
53	Lr-270	JN581334	F: ATCAAAGTGTGGTGCAAGGTTT	(AC)20	57	185

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			R: TCAGGTGGAAAATAAGTGTGGA			
54	Lr-271	JN581335	F: TCACAGCAGGACTGAAGAAATG	(CA)8	57	206
			R: TGTAGGGCGATAGAAAGTACGG			
55	Lr-271d	JN581335	F: TCACAGCAGGACTGAAGAAATG	(CA)8	57	206
			R: TGTAGGGCGATAGAAAGTACGG			
56	Lr-278	JN581342	F: TAACGCTTATTCCAGTCCCAGT	(AC)19	57	207
			R: CGCAGCCAATAGTACACCTCTC			
57	Lr-278d	JN581342	F: TAACGCTTATTCCAGTCCCAGT	(AC)19	57	207
			R: CGCAGCCAATAGTACACCTCTC			
58	Lr-279b	JN581343	F: GGTCCCGTTTCAAGCTCTTAC	(CA)9	57	201
			R: CGCCACCAAATGACTACAAA			
59	Lr-297	JN581360	F: ACCACAGCCGTATGCCTAAC	(GT)19	57	201
			R: GACTCCATGCGTCACACAAC			
60	Lr-298	JN581361	F: TGGTCCCATAACGTGATGAAT	(AC)17	58	246
			R: GAAAACAGGCATCTGAACACAA			
61	Lr-300	JQ862039	F: TTAGTGGCATCCTGCTCTCG	(GAG)9	58	98
			R: GGAGTGAGTTTGAGCGATGG			
62	Lr-302	JQ862289	F: GTCAATCAGGAATGAGGGCTG	(CAT)12	58	98
			R: GTGACCAGTAAAGAACTGACCTG			
63	Lr-303	JQ862042	F: CTTCCCTGCGAGACAGACG	(GA)15	56	98
			R: ACAGATACGCTTCCCCTGTG			
64	Lr-306	JQ862045	F: CCAAGACCGCGCATTACTTC	(GAT)9	56	99

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			R: TTGAGATTTGTCTGACCCCG			
65	Lr-313	JQ862052	F: TTCCGCACTTTTCCGAATCC	(ATC)11	59	102
			R: ACGACCTGGAGGATGTTGAC			
66	Lr-336	JQ862075	F: TATCCACCTGTCATCCGTCC	(CCAT)7	59	109
			R: GGGTCGAAAATGAGTGGGTG			
67	Lr-347	JQ862086	F: CAGGCCAAAGTGACAGGC	(AGG)9	59	112
			R: TGGCTCACACATGCATTAGG			
68	Lr-351	JQ862090	F: GCACCTGAAGGTTGCTATGG	(AC)11	59	114
			R: GGTTTGAGCGGCATTGTGTG			
69	Lr-352	JQ862091	F: ATCCGCTGGTTTATGTGTCG	(TGA)13	59	114
			R: ATAGACTGCAGCACCTCGTC			
70	Lr-358	JQ862097	F: TTCTTGTCTCTTTTCCATAGTCAG	(GGA)7	56	117
			R: AGACCACAAATGCCTGCAAC			
71	Lr-361	JQ862100	F: AGCTGCTTGTAAGACAATTTTTGC	(AC)15	56	118
			R: GCTGTTTTCTGACCTCTCACC			
72	Lr-362	JQ862101	F: CAGCCAGAGCCTGAAAGATG	(GAT)10	56	118
			R: AGCAGTTTCTTTGTCCTCTTCG			
73	Lr-363	JQ862154	F: CAGGACTCGACCCTCATCTC	(CAT)15	56	118
			R: GAGGGACATGGTGGGAAGC			
74	Lr-381	JQ862238	F: CTTCTTCCGTCAGTACACGC	(GAT)9	57	122
			R: ATCAAATGCCCAAGCAACCC			
75	Lr-382	JQ862121	F: GCAGCCCATCTGATCTGGAG	(AG)15	58	122

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			R: TTTTGGTAGTGCCTCAACGG			
76	Lr-383	JQ862132	F: TCATTTAACACAGAGAGAGAATGAG	(AG)21	58	122
			R: GATGTGTTCTGGCGTCACAG			
77	Lr-384	JQ862123	F: ACACCTTTGAGCTCTTCTTGC	(ATC)15	58	122
			R: TCAGGGGCAGAGTGATGATG			
78	Lr-388	JQ862127	F: AAAGGTGAGACACTCGTTTTC	(TG)16	58	125
			R: TAGATATGGCCAACCCACGG			
79	Lr-393	JQ862132	F: ACAAGCTCATTTAACACAGAGAGAG	(AG)21	58	126
			R: GATGTGTTCTGGCTCACAGG			
80	Lr-394	JQ862133	F: AAATGCATCAGTTCCGCTGG	(GAG)8	58	126
			R: GAGCGGACAGGAGGTAACG			
81	Lr-401	JQ862140	F: ACGATTTACGGATGATGCGAG	(AG)20	58	128
			R: TTCCCTGCTTCCCTATGAGC			
82	Lr-402	JQ862141	F: CCTAATTGTTAATCAGAACTCTCTTCC	(GA)14	57	129
			R: TCTGTTCACTCAATCACGTATGC			
83	Lr-405	JQ862144	F: TACAGAGAGGAGCGCGTATG	(GAG)7	57	129
			R: GCAGTTGTTTAATCCCTCCCC			
84	Lr-406	JQ862145	F: GCCGTGAACTCCTCAAATGG	(TG)15	56	129
			R: TGAGTGAGTGATGTTAGTCCG			
F	Lr-415	JQ862153	F: CTGCAAACAGGTGAGGTGAG	(AG)19	58	132
			R: ACCATTATAAAGACACGGAGCC			
86	Lr-416	JQ862154	F: CAAACACAGCAGGACTCGAC	(CAT)15	58	133

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			R: TTGATGAGGGACATGGTGGG			
87	Lr-419	JQ862157	F: CAGACGAGTGTTTTCGGTCG	(ATC)8	57	133
			R: GGTGATTGGACATGGACACG			
88	Lr-420	JQ862158	F: TGTGAGCCAAGATCGGAGAG	(TCC)7	57	133
			R: ACTGACGCTCATGCTAGAGG			
89	Lr-424	JQ862162	F: AATTTGAGGTCGGTTCAGGC	(CAT)7	57	135
			R: TGGCCTCATAAAGTCTTGCG			
90	Lr-425	JQ862163	F: GTGCGCAGATAGCTCCAAAG	(TGA)9	56	136
			R: CAGTCTCTAGGGAACCCAGC			
91	Lr-427	JQ862165	F: AAGTCTCCATCCATGCTGCC	(ATC)11	55	137
			R: AGCAATGTGAAGCCCAAGTC			
92	Lr-432	JQ862170	F: GGTTGGGATAGGGGACAGAC	(GT)13	55	138
			R: GCTCCCCAGAATGCCTAAAC			
93	Lr-437	JQ862175	F: TTTTGCTCAAGTGAAGCCCC	(AG)12	58	142
			R: ATCTCACGTGTCGCTCTCTG			
94	Lr-442	JQ862179	F: ACTGTCTTCAGTTTGTGCTGG	(CAT)8	58	143
			R: AGATGATGGACTCATGAAGCAG			
95	Lr-446	JQ862183	F: GGAGAACGCATGTTTGTGTG	(TG)13	58	146
			R: AGGTATACAAGGCACGCTCG			
96	Lr-447	JQ862184	F: TGGCCAGAGAATGGGTATCAG	(GAG)9	56	146
			R: CCCAAGCCAAATGTCTCCTC			
97	Lr-451	JQ862188	F: CCCCATGCATCTTTCTGTCG	(TCTA)7	56	148

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			R: AGTTTAAGTTTGGATGGTTTTCAGTC			
98	Lr-452	JQ862189	F: ACAAAGAAAAGCTCAACAAAGTCAG	(GAG)13	56	148
			R: ACCGGCTGGAGATAATGGTC			
99	Lr-456	JQ862193	F: AGCTCATATAATCCTCACACACG	(TGT)10	55	150
			R: GCCCATGTGACCAATGAAGG			
100	Lr-458	JQ862269	F: TCTGAGATGTGTCTGGCTGG	(GAG)10	55	151
			R: CTCCTCTTCCTCCTGTGAGC			
101	Lr-461	JQ862198	F: AGACGAGTTTCTGGGAGCTG	(TCA)12	56	151
			R: GGCTGACCCATGAGCAATAG			
102	Lr-463	JQ862200	F: TGTGAATGAAGCAGGACAACAG	(CA)17	56	151
			R: AGATTTGTAGCCCTGTGGGG			
103	Lr-464	JQ862201	F: GCTTTCCGATAACGTGGCAG	(GA)16	56	151
			R: ACTGGTTTAGTTCTTCATCTTCTCTC			
104	Lr-467_F	JQ862204	F: GCACAAATCAGCAATGCGTC	(AC)11	58	154
			R: GATGCTCTGTGTGAACGCTG			
105	Lr-470_F	JQ862207	F: GAGACAGATGGATCGAACGC	(CA)12	59	157
			R: TAATGTCCGTGGTCTGCTGG			
106	Lr-475	JQ862212	F: AAGAAGACACAGGCTAGACG	(GAG)7	56	161
			R: AGCGCTTGGGAACAATTAGC			
107	Lr-476	JQ862213	F: ATGGGGCGATAGAGACACAC	(ATG)9	57	162
			R: GTTCCTGCACTCACATCCAC			
108	Lr-476d	JQ862213	F: ATGGGGCGATAGAGACACAC	(ATG)9	57	162

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			R: GTTCCTGCACTCACATCCAC			
109	Lr-477	JQ862214	F: CATGGGGGCGATAGAGACAC	(ATG)9	57	162
			R: GTTCCTGCACTCACATCCAC			
110	Lr-485	JQ862222	F: TCTACAGCACACTGACCCTG	(TC)12	57	170
			R: AGTAACCGACAATCTGTGGC			
111	Lr-492	JQ862229	F: AGCTGCAGAATCCCTCTCTG	(GATA)19	58	173
			R: TATCTGTCTGCCCATCCGTG			
112	Lr-493	JQ862230	F: TGTTGGTTCTTTACGGTGTGG	(CAT)11	59	174
			R: ATCAGACACGCCCACTGATG			
113	Lr-499	JQ862236	F: GCCATAGACTTCCAGAGCGG	(GAA)8	57	178
			R: GCCCAACGTTTGCTGTTTTTC			
114	Lr-501	JQ862238	F: ATCAAATGCCCAAGCAACCC	(CAT)11	57	180
			R: TCGGAACACACCGCATTAAC			
115	Lr-506	JQ862243	F: TGTTTCCTGTGAGGGCTAGG	(GT)14	57	186
			R: GCAGTGCTGAGGTTAGTCTG			
116	Lr-507	JQ862244	F: GGTTCACAAAATGTGGCTGTG	(AC)15	57	186
			R: GGTTGATTCGCTCGTGTGTG			
117	Lr-513	JQ862250	F: AGCTGCAGGAAACGAGTCTG	(TGA)14	57	192
			R: GATGTCTTCTCGTGCTTGGC			
118	Lr-517	JQ862254	F: CTTGACAATTAATAAACATGTGAGC	(GAT)10	56	195
			R: TCAGTTCATCATGGTGCTGG			
119	Lr-519	JQ862256	F: AGCTTGGTCAGGGCTAGAAC	(CTC)7	56	198

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			R: AGAGACTGCAGCTTTCCCAG			
120	Lr-526	JQ862263	F: GAGAGAAGGTGGGCTCTCTG	(ATG)14	56	206
			R: CGTTTGACCGTCTTATCGCC			
121	Lr-532	JQ862269	F: TTGGACCGATCGTGTACCTC	(GAG)10	55	213
			R: CTCCTCTTCCTCCTGTGAGC			
122	Lr-539	JQ862276	F: AGTTGGCATTTCCATCCAGAC	(ATC)11	56	230
			R: TCATGTGCTCGGTGATTGTG			
123	Lr-540	JQ862277	F: TTTAGCCCTCACGCGGTATG	(TGA)10	55	230
			R: GGAAAGACAGGACGCTGAAC			
124	Lr-541	JQ862278	F: GACGCCATCGTTGTAGTTACC	(GA)12	57	231
			R: TCGAGCGGACATGAGAAGAG			
125	Lr-545	JQ862282	F: ACTCCTCCTGACACCTTGAG	(TGA)9	57	236
			R: ACGAACAGTGCAAAGACGTG			
126	Lr-547	JQ862284	F: CTGTAGATGCTGGTCTGGGG	(CAT)10	57	238
			R: ACCTTACATTGGCTGTTGGTC			
127	Lr550	JQ862237	F: ACTGAGAAAATGCTGCAACAAC	(ATCT)10	56	180
			R: CTGGATGGGTGGACAGACAG			
128	LF-1	JQ838157	F: GAAAGCTGCTCGTCCTTGAA	(AG)19	58	192
			R: CTCGGGATGAGAGCAGAAAC			
129	LF-4	JQ838159	F: GGCCAGTGTGACACAAACA	(CT)12	58	227
			R: GTCCCGGAGTCTAAAGACGAAC			
130	LF-7	JQ838162	F: TAGCAGACCTGCACTGAGAAAG	(AG)20	58	179

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			R: TCTCCCTGGTCTTTTCCT			
131	LF-8	JQ838163	F: GTGAAGCAACGACTTCAGAGAG	(GT)6	58	216
			R: CCAGAAGACCATAGCAACCAC			
132	LF-9	JQ838164	F: GAAGTGACGGTCGCTGTTTC	(AG)27	58	223
			R: GGGATTGTCTGTAGTCCGTAGG			
133	LF-12b	JQ838167	F: AGGAAACACAGTGAGTGCAGAG	(CA)10	58	206
			R: GAGAGAGCGATGCACAATCA			
134	LF-13	JQ838168	F: GCCTCATTCCCAGTTACATCAG	(CTG)4	58	186
			R: TCCAGCAGAGAGAGAGAGAGAGA			
135	Lr-est-50	Unknown	F: TCGTATTAGTGGTCGTGTCTCG	Unknown	57	156
			R: TCCTCTCAAAGGAACAGAATGC			
136	Lr-est-51	Unknown	F: GCTTGGGCTAATACCAAGTGTT	Unknown	56	238
			R: GCTGTTATAGGGAATGACTTCTGG			
137	Lr-est-52	Unknown	F: ACGGACTCAGAATAACCGTGAC	Unknown	56	206
			R: AACTTTTGGTTCCGTCTGACAC			
138	Lr-est-53	Unknown	F: GTCAGAGGGTGATGAAAAGAG	Unknown	56	190
			R: ACGAGAGGGGTCTGATATCGT			
139	CC-46	Unknown	F: CTCTCCCTCTACCAGGCATTTT	(TC)6	60	125
			R: GTCAGGTGTTGAAGCTCTTTCC			
140	CC-42	Unknown	F: CTGGCCTGTATCTCGCTCTG	(GT)13	61	141
			R: TACACTTGACTAACCCGGACCT			

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