RESEARCH ARTICLE

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 the Indian subcontinent, was developed by placing 68 microsatellite markers generated by a simplified method. The parents and their F_1 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 3087.9 cM. This genetic linkage map may facilitate systematic searches of the genome to identify genes associated with commercially important characters and marker-assisted selection programmes of this species.

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Introduction

Labeo rohita, commonly known as 'rohu', is one of the major Indian carps used successfully in commercial aquaculture in the Indian subcontinent at least for the past six decades. Integration of modern molecular biological tools, along with conventional breeding programmes, provides speed, reliability and sustainability in producing improved varieties. An ongoing selective breeding programme in rohu, which started in 1992 at Central Institute of Freshwater Aquaculture, has achieved 17% higher growth per generation (Mahapatra *et al.* 2007), whereas markers needed to achieve further accelerated genetic improvement in rohu have just begun (Patel *et al.* 2009; Robinson *et al.* 2012; Sahu *et al.* 2013). 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; Dor *et al.* 2014; Hermida *et al.* 2014; Keong *et al.* 2014; Hollenbeck *et al.* 2015) 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 and production of microsatellite markers (Perry and Rowe 2011). In this study, we report: (i) the development of microsatellite markers; (ii) the use of these markers in the construction of a genetic linkage map of rohu; and (iii) 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, 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

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Titanium pyrosequencing platform, and high quality reads having more than 200 bp were taken for further processing. QDD program to find microsatellite sequences (a minimum of six repeat units for dinucliotide and trinucleotide, five for pentanuecleotide and hexanucleotides and four for heptanucleotides, 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, USA) was subjected to Sanger sequencing using Big Dye terminator ver. 1.1 cycle 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 *et al.* 2008).

Mapping population

Single F_1 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 collecting 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–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 is as follows: 493 loci were developed in the present study, 47 loci were previously developed in our lab (Das *et al.* 2005; Patel *et al.* 2009), 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 containing 1× *Taq* polymerase reaction buffer with 1.5 mM $MgCl_2$, 200 μM dNTPs, 5 pmol of both forward and unlabelled reverse primers, 0.25 U of *Taq* DNA polymerase (Bangalore Genei, Bengaluru, India) and 20 ng 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 30 cycles of 45 s at 94°C, 1 min at the annealing temperature of the corresponding primer (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/) and 2 min at 72°C and, finally, an extension of 15 min at 72°C. One microlitre PCR products and 0.1 μL GeneScan^{TM}–500 LIZ^{TM} size standard (ABI) were added to 9.9 μL of Hi Di^{TM} formamide. The mixture was subjected to genotyping on an ABI 3770 sequencer (Applied Biosystems), and GeneMapper ver. 3.7 software (Applied Biosystems) was used to edit and analyse data.

Microsatellite segregation and linkage analysis

Genotype data of parents and 92 progeny at 140 microsatellite loci were subjected to goodness of fit of the observed-to-expected allelic ratios using the chi-square test. Markers heterozygous for at least one of the parents were defined as polymorphic. Markers segregating in Mendelian ratios were used for construction of map. Genotype configuration of markers was categorized into four expected segregation types: 1:1:1:1 ratio type (♀ × ♂: AB × CD or AB × AC), 1:2:1 type (AB × AB), 1:1 ♀ type (AB × 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 F_1 outbred 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 ver. 2.2 (Voorrips 2002). On the basis of consensus map, expected genome length was obtained by the following two methods: (i) 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); (ii) 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 (G_e) of rohu. The observed genome length (G_o) was calculated as the total length of the framework map. The map coverage was determined by G_o/G_e. Comparison of meiotic recombination rate among parents was performed by taking common marker pairs.

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 dinucleotide, trinucleotide and tetranucleotide 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 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 in 748 (40.6%) dinucleotide, trinucleotide and tetranucleotide repeat motifs within the sequence reads. Of these, 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 F_1 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 five resulted in 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 × CD (12 markers) or AB × AC (30 markers), 36 with AB × AA or CC and 49 with AA × 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), five of which contained four markers, eight contained three markers and 12 LGs had two markers. The consensus framework map consisted of 25 linkage groups (figure 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 two to four. The average marker spacing was 34 cM. LG1 had the largest average interval of 41.15 cM and LG25 had the smallest average interval of 2.35 cM. The average estimated genome length was 3087.9 cM for the consensus framework map of *L. 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 female map (545.9 cM, in comparison with the male map (348.8 cM). Adding the length of the interval beteen the loci, rendered a total length of 96.7 cM and 162.2 cM in the male and female map, respectively. Thus, the recombination rate in 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 are more complex in full-sib families of out-breeding species than in progenies derived from homozygous parents. For example, markers may vary in number of segregating alleles, one or both parents may be heterozygous, markers may be dominant or codominant and, usually, the linkage phases of marker pairs are unknown. In the 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 $2n = 50$ 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 (Santana *et al.* 2009; Martin *et al.* 2010; Davey *et al.* 2011). 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 (Abdelkrim *et al.* 2009; Csencsics *et al.* 2010; Kircher and Kelso 2010; Yu *et al.* 2011). Various microsatellite development protocols based on partial genomic library enriched for microsatellite are available in the literature (Liao *et al.* 2007; Pardo *et al.* 2007; Abdelkrim *et al.* 2009; Wang *et al.* 2010). However, marker development following Sanger sequencing has shown to be more effective (Malausa *et al.* 2011). In the present study, both Sanger sequencing as well as 454 GS FLX Titanium pyrosequencing were employed to develop microsatellite markers. Next generation sequencing resulted in only 3.6% sequences suitable for primer designing compared with 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 honey bee (*Apis melira*) and zebrafish (*Danio rerio*), only 1.2–5.4% sequences could be used to design primers for microsatellite marker development by 454 FLX titanium pyrosequencing (Saarinen and Austin 2010; Malausa *et al.* 2011).

Linkage analysis

Like many other aquaculture species, mapping resources such as purebred lines, near isogenic lines and inbred lines are not available for *L. rohita*. Pseudo-test cross mapping strategy to construct linkage map has been successfully employed in several 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 F_1 pseudo-test cross 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

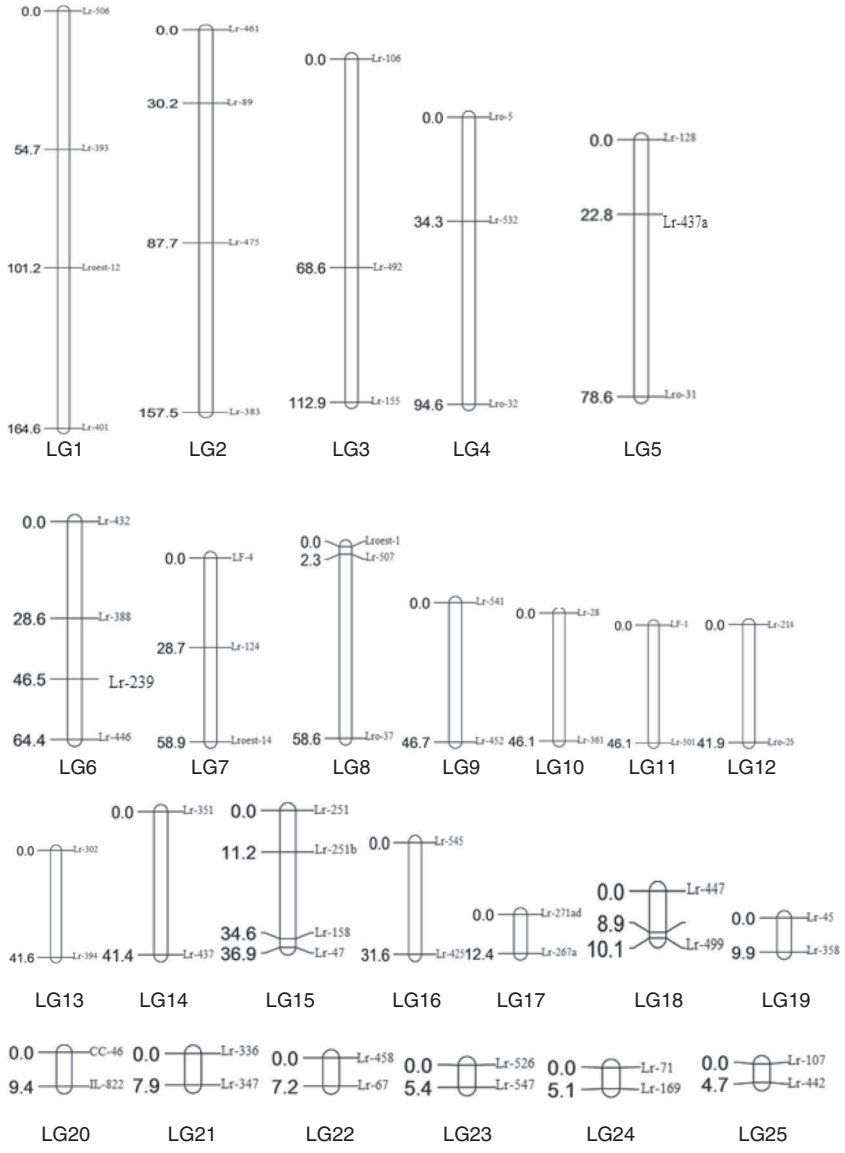

Figure 1. Microsatellite-based consensus genetic linkage map of rohu using F_1 full-sib family. Distances in centiMorgan (cM) Kosambi are indicated on the left side. Acronym of the markers are on the right side.

markers like AFLP (Li *et al*. 2012). This study shows that the microsatellites are effective markers for linkage mapping in *L. rohita*, as it 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 observed in the present study compared with 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 frequent problem encountered 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, *L. rohita* constructed using an F_1 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 that 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, crossover 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, the number of markers placed in each LG is much less than the SNP-based linkage map reported earlier (Robinson *et al.* 2014). Based on the current linkage map, the average genome length of rohu was estimated to be 3087.9 cM, which is much higher than the length estimated by Robinson *et al.* (2014). This might be due to a 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 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 with 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 female map when common informative markers were taken into account (figure 2). Adding map distances for common marker 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

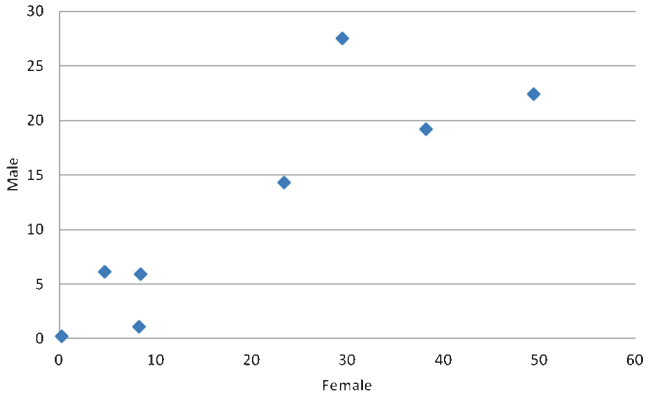

Figure 2. Differences in recombination frequency in male and female parents. Recombination distances are in cM. Recombination distances of male are in y-axis and female are in x-axis.

markers were 1.67:1. Though the molecular mechanism responsible for differences in recombination rates between the two sexes is not well understood, higher recombination rate in female has been observed in several teleost species. For instance, the female : male rates are 8.26:1 in the Atlantic salmon (Johnson *et al*. 1987; Moen *et al*. 2004, 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 was 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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