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SSLP-based Linkage Map Construction in Black Tiger Shrimp (*Penaeus monodon* Fabricius)

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ABSTRACT: Microsatellites represent an abundant source for genetic markers in eukaryotic genomes. We developed microsatellite or simple sequence length polymorphism (SSLP) markers for the black tiger shrimp genetic improvement programs. Construction of a genetic linkage map was initiated for *Penaeus monodon* using 57 SSLPs, EST and SCAR markers with 76 individuals of an F1 inter-cross family. Segregation markers were scored and analyzed with the JoinMap 2.0 program. A total of 50 markers, confirmed with expected Mendelian segregation ratios of 1:1 or 1:1:1:1 (χ^2-test at the α = 0.01 level), were grouped with a LOD score of 5.0. Results showed that 27 loci formed 9 linkage groups and there were 23 unlinked loci. The largest group had 7 markers spanning 29.5 cM. The average distance between markers was 3.8 cM. This linkage map covered a total genetic distance of 103.6 cM.

KEYWORDS: Linkage map, *Penaeus monodon*, SSLP markers.

INTRODUCTION

Previous studies suggest that microsatellites are abundant and widely distributed in the genomes of shrimps.[1-6] This class of co-dominant DNA marker detects higher levels of allelic variation than randomly amplified polymorphic DNA(RAPD) markers, and can be easily assayed by the polymerase chain reaction (PCR). However, difficulties encountered in the isolation and analysis of microsatellites from penaeids limits the number of microsatellite markers available for linkage mapping and quantitative trait loci (QTL) detection studies.[3]

A gene map can be used for the identification of QTLs controlling economically important characters, such as growth and disease resistance, and the development of marker-assisted breeding programs for strain improvement. A linkage map based on Amplified Fragment Length Polymorphism (AFLP) has been reported for *Penaeus japonicus*.[3,7] Efforts have also been made to develop a preliminary genetic linkage map for the most important cultured species, black tiger shrimp, *P. monodon*.[8]

In this study, we aimed to construct a linkage map of *P. monodon* using microsatellite or simple sequence length polymorphism (SSLP) markers[9], a *P. monodon* expressed sequence tag (EST) marker (an EST which matched a gene encoding an anti-microbial peptide of *Litopenaeus setiferus*; Pmpsb) and a SCAR marker (an AFLP-derived PCR marker from a low- and high-growth *P. monodon* polymorphism; Tag4), with an F1 inter-cross family.

MATERIALS AND METHODS

Production of reference family and DNA isolation

One F1 inter-cross pedigreed family was produced by artificial insemination from the research project " recurrent selection of black tiger shrimp (*Penaeus monodon* Fabricius) under low salinity culture" funded by the National Research Coucil of Thailand. The pleopods of parents were preserved at the time of spawning. The offspring was cultured under low salinity conditions (5 ppt). One hundred samples were randomly selected, with offspring being preserved in 95% ethanol for future DNA preparation. Total genomic DNA was isolated from alcohol-fixed tissue via DNATrap (DNATEC, Nakorn Pathom, Thailand.), according to the manufacturer's instruction.

SSLP genotyping

A total of 57 SSLP (Table 1), 1 EST and 1 SCAR

Table 1. Microsatellite markers of *Penaeus monodon*.

Locus [a]	Clone	GenBank Acc. no	Repeat type [b]	Repeats	Primer sequence
DPm101	B0311	AY189723	C	$(GA)_{48}+(GACA)_7+(GAAA)_6$	ggtcttgcttccctaacctgtcc attctcttcatctttccgagtctgc
DPm103	B14-2/6	AY188966	C	$(GA)_6+(GACA)_4+(GATA)_{21}+(GTCA)_{31}$	tgttcccctcactgtgtcctac gggaagcgcctgtagagtagag
DPm104	T-221	AY188967	C	$(CAGT)_{23}+(TC)_{11}$	aggacctgcatttgtgtcg atggcgagacaaggttcg
DPm105	B14-12/1	AY188968	C	$(TATC)_4+(TCTG)_6+(CAGT)_{10}+(GAGT)_3$	tgcatacctaacgtacctacaca aagctgaatgcaggtcgagt
DPm107	T-419	AY188969	C	$(CTC)_9(CAT)_{31}(CATCATC)_3+(CATCAAT)_3+(CAT)_{21}$	ccagtgaaggatatgaggaagc gccacacgaaacctctactgc
DPm108	T-518	AY188970	C	$(GA)_{10}(GACA)_3(GAGACA)_4(CATT)_3+(CATT)_3$	cggatgaactgtcagtggac ttagccacggaagaagcact
DPm109	T-603	AY188971	C	$(CAGT)_{42}(CACT)_5$	tttaaggactttcgcaatgg cgcattccaactgctcatag
DPm110	T-611	AY188972	C	$(TTC)_{10}$ TTTT $(TTC)_{17}(TAC)_{25}$	agtcaccaaggaatcggagt taaatcgtcggcaaatgtca
DPm111	T-607	AY188973	C	$(CAT)_{12}+(CAT)_{25}(CAA)_{10}+(CAG)_9+(CAT)_9$ $(CAG)_7$	ttcagggattctctgctgga gcgagataaggatgctggag
DPm112	T-614	AY188974	C	$(CAGT)_{48}+(GAAT)_4$	ctgtgtaatgggcttgtgga aaagcagcaacgagaggaag
DPm113	B12-/8	AY752996	C	$(CAGT)_{48}+(GAAT)_4$	ttattccctggagccaagac cacgtgataatgcagtagtttcag
DPm114	Ct-108	AY752997	C	$(TCC)_8+(CAT)_{25}$	ccagcgcattcttccttatc tccagaaaagggatgggaat
DPm115	ZT-112	AY188975	C	$(CTC)_9(CAT)_{85}$	catgtaccgcccactcatc gccaacacgaaactctactgc
DPm116	T-507	AY752998	C	$(GACT)_5+(GACT)_3+(GACT)_5+(GACT)_{15}+$ $(GATT)_3+(GACT)_{11}+(GACT)_3$	cttgggggaagaatatttctgag gagagaaaaggaagatgagacga
DPm117	T-1309	AY188976	C	$(GCAC)_4+(CA)_4+(CA)_{46}+(CA)_5+(CA)_3+(TC)_3$	tatgaagataagacgcgctcaag cgtacatttgttgtttattgaggag
DPm119	T-1307	AY188977	C	$(GA)_3+(AG)_8+(GA)_3+(GACT)_4+(GACT)_4+(GACT)_4+$ $(GACT)_3+(AGAC)_3+(ACAG)_3+(AG)_3+(GACA)_3+$ $(ACAG)_4+(AGAC)_5$	attggaaaagcttgaggatgg atgatgaaatgagtcgactgtca
DPm120	T-1401	AY188978	C	$(AG)_{11}+(TGAC)_4+(TGAC)_{24}$	ttatccgtatagccgcgttatc ttacaggacctgcatttgtgtc
DPm121	T-1410	AY188979	C	$(GTT)_3+(TCA)_{21}+(CAT)_3+(TGA)_3$	aatcactgacttgcacaacct cggtgacgttataaggcaagt
DPm125	T-706	AY188980	C	$(CAT)_{13}+(CAT)_{25}+(CAA)_{20}+(CAT)_4+$ $(CTTCAT)_3+(CAT)_4+(CATCAG)_3+(CAG)_{10}+(CAT)_9+$ $(CAG)_7$	ggatttagagagtgggctgtctt gagataaggatgctggagattga
DPm127	T-1103	AY753003	C	$(GTT)_3+(TG)_3+(CA)_3+(CT)_7+(CT)_{10}+$ $(CTCG)_5+(CT)_{14}+(TC)4+(CA)_{24}$	gtggttgttgcaaatgtggt ctgaaatcgtgcttttgtgc
DPm130	T-417	AY188982	C	$(GTC)_{25}+(GAA)_{17}$	gaatcggagtcggtgtcttc caagcaaacaaccgaacaag
DPm131	T-812	AY753005	C	$(GC)_{11}+(GACT)_{24}$	cgttatccgcacgttattcg aggacctgcatttgtgtcg
DPm132	T-1503	AY753006	C	$(GACT)_{21}+(TC)_5$	ccgtaaacatccttctcaacg atgtagtcatcgccacaaactcc
DPm137	T-2106	AY753010	C	$(TC)_{23}+(CA)_7$	tgttggcattacttctgcatac gtcgggctcataaagtttcgta
DPm138	T-1603	AY753011	C	$(CATA)_{24}+(CATA)_{35}+(CA)_5+(CA)_8$	acatccattcacagacatacgc gatactctttacaactgcgacca
DPm139	T-1617	AY753012	C	$(CAGT)_{23}+(TC)_{12}$	ttacaggacctgcatttgtgtc ttatccgtatagccgcgttatc
DPm140	T-1903	AY753013	C	$(CAT)_{26}+(CAA)_9+CAG)_{10}$	ctggatttagagagtgggctgt gagattgaatagcggatgcac
DPm141	T-1604	AY753014	C	$(CAGT)_3+(GACA)_{84}$	tgctgactgattgactgatct ccttcgtccccsatctaatcc
DPm142	T-1708	AY189725	C	$(CACG)_{11}+(CA)_{21}$	tgagatagctaatttaggtgtaccttc ggacaagaaataacacacttaaaggtc

Table 1. *Cont'd.*

Locus [a]	Clone	GenBank Acc. no	Repeat type [b]	Repeats	Primer sequence
DPm145	T-1807	AY753017	C	$(GCT)_7$+$(GAT)_9$	ggagattgaataacggatgcac
					ctggatttagagagtgggctgt
DPm148	T-1904	AY753020	C	$(GA)_{44}$+$(GACT)_{17}$	ttatccgtatagccgcgttatc
					ttacaggacctgcatttgtgtc
DPm151	T-2801	AY753023	C	$(TC)_9$+$(CAT)_{12}$+$(CAG)_7$	cgttagtgctgtttcctgtgtc
					gtgatggtaatggtggtgatga
DPm152	T-2803	AY753024	C	$(CA)_3$+$(TA)_6$+$(GA)_{38}$+$(GACT)_{36}$	actgacactcactcgtgcacttg
					gattttattcgtacgtgacttcag
DPm153	T-516	AY753025	C	$(CATCGT)_7$+$(CAT)_{23}$	ttacaggacctgcatttgtgtc
					ttatccgtatagccgcgttatc
DPm154	T-1814	AY753026	C	$(CAGT)_3$+$(TC)_{11}$	tttatttgtgcttgactggcact
					atggtgatgatgattgatgacg
DPm203	B2	AY187720	P	$(CA)_{46}$	ctcccattaatcctgtcctgaa
					tgttgggggatagggaatagat
DPm205	CT-106	AY188983	P	$(TC)_7$	acgacgacgtcaaagtctca
					cagacaaacggacaaagacg
DPm206	T-520	AY753030	P	$(CA)_6TA(CA)_{30}TA(CA)_9TA(CA)_8AA(CA)_8$	ctttgctttgtgctacataccc
					ggggaatcaagtgaacaagg
DPm207	T-809	AY753031	P	$(CA)_{45}$	ctgggaagagcgtggtgtag
					aggcataatgaagggcatgg
DPm208	T-620	AY753032	P	$(CA)_3$	tattccacttgaccattacacga
					gccgatagtgttgtttggtagag
DPm210	T-1105	AY753034	P	$(TC)_{46}$	tctaattacacggacacacatcg
					gcaagaatctcctaaaactctgc
DPm211	T-408	AY753035	I	$(TC)_9$+$(GA)_{33}$	atcacagcgtaatgaaagcctg
					gacctgtaaggatcccagtcgt
DPm212	T-1305	AY753036	P	$(TA)_3$	tcggagtagttaagggtgatttg
					gccacgccattagagactaca
DPm217	T-1607	AY189726	P	$(CA)_8$	tctattgtctgccagtttgtcc
					tagcacgggatttatgaagtga
DPm218	T-1711	AY189727	P	$(TC)_{19}$	attccgcaatatatcggtttcc
					aatgtttccatttcatgcttcg
DPm219	T-1808	AY753041	I	$(GA)_{13}$	taaatgtcagccctgaagctca
					tcccaacgattactcaaacctc
DPm223	T-2504	AY753045	P	$(TA)_5$	tcagaactacgatatggattataggtg
					gtaggtagattccatggggttg
DPm302	T-513	AY753051	P	$(CAT)_6GT(CAT)_{25}$	gctccgtttaatgaggtcgt
					cgcgtatcgatgaatgagag
DPm303	T-609	AY188984	I	$(TAC)_9$+ $(TAC)_{24}$	tgccttgtattttgacgatcag
					ttggagtagcaacagcggta
DPm308	T-1413	AY188985	P	$(CAT)_9$	actcgcgtttactttccttcg
					cgagaatcacgaagaagatgc
DPm310	CT-102	AY753056	I	$(CAT)_{16}$	aaggctgatttatcgcttgc
					ttcccgtgaataccaatgc
DPm313	T-1809	AY189728	P	$(GAT)_{23}$	tgggaaattatgtaagggctgt
					attcctccacacgacttactgc
DPm315	T-1908	AY753060	I	$(CAG)_6$	aagccacagaatctgaacctaga
					aaccggtaggctcccaagtc
DPm316	T-1912	AY753061	P	$(GAT)_{11}$	ggagtagacccgagagtgtcag
					acctccactggtaattcctcct
DPm317	T-2603	AY753062	P	$(CAT)_{25}$	tatttcgtagacctttggcaca
					ccggtgaaggtaaacaatacca
DPm401	T-904	AY753063	P	$(CAGT)_{13}$	tcaggttatgaagagagaatgga
					cgttgtgtttaacccgaga
DPm402	T-622	AY188987	P	$(CAGT)_{19}$	ccactctaactccgccagtc
					tccctaccccactatcatcg

[a] Microsatellite or SSLP locus: DPm = DNA Technology Laboratory *Penaeus monodon*.
[b] Repeat types: P = perfect repeats, I = imperfect repeats and C = compound repeats.

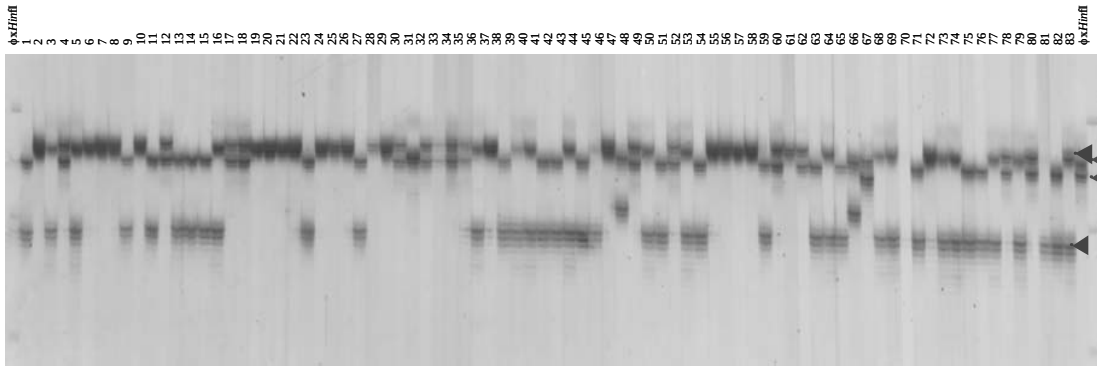

Fig 1. Segregation patterns of a *Penaeus monodon* family amplified by DPm302 SSLP marker. The arrows indicated alleles from parents (sample no. 82 and 83) segregated into progenies (sample no. 1-81; except 31,47, 65, 66, 69).

markers were used to genotype the parents and 79 progeny individuals. The reaction was performed in a 10 ul mixture containing 5 ng genomic DNA, 2.5 pmol of each primer, 200 mM dNTP, 90 mM Tris-Cl (pH 9), 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, and 0.2 unit *Taq* DNA polymerase (Promega, Madison, WI, USA). Amplification was carried out on a GeneAmp 9700 thermal cycler (Applied Biosystem, Foster City, CA, USA) with the following PCR conditions: 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final cycle of 72 °C for 5 min. PCR products were added to an equal volume of stop solution (98% deionized formamide, 2 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 94 °C for 3 min. A 3 ml aliquot of each PCR product mixture was analyzed on a 4.5% denaturing polyacrylamide gel. DNA fragments were visualized by silver staining.

Linkage analysis

The notation of segregation markers follows that of Wu et al.[10] using different characters to denote different alleles of a marker locus including '0' for a null allele. The left of the 'x' is the genotype of the mother, and the right of the father. Segregation types "ab x ac" and "ab x cd" are used for markers segregating in a 1:1:1:1 ratio in the progeny; "ab x ab" is used for markers where parents were both heterozygous with the same two alleles and the segregation ratio of 1:2:1.

Single-locus analysis, grouping of markers and mapping were performed with JoinMap version 2.0[11], which permits linkage analysis in outbred progenies

Table 2. Summary of the segregation patterns applied for mapping analysis.

Parent			Progeny			No. of loci
Band		genotype	Observed band	Genotype	Segregation	
Dam	Sire					
—		aa x oo	—	a, o	1 : 1	4
	—	oo x aa	—	a, o	1 : 1	3
—		ab x oo	—	a, b	1 : 1	2
—			—			
	—	ao x bo	— —	ab, a, b , o	1 : 1 : 1: 1	5
—			—			
—		ab x cd	— —	ac, ad, bc, bd	1 : 1 : 1 : 1	38
	—		—			

involving markers with different segregation types. Goodness of fit of observed-to-expected allelic ratios was analyzed using the c^2-test. Markers were defined as polymorphic alleles if they did not significantly depart from Mendelian ratios at the a = 0.01 level. Alleles with significant segregation distortion were discarded prior to the linkage analyzes. Data were then analyzed by using the "cross pollination" ("CP") population-type option according to the JoinMap program's instructions. Map distances expressed in centiMorgans (cM) were calculated by the Kosambi function.[12]

Results

Marker segregation

Of a total of 59 marker loci genotyped, 52 loci (88.1%) were clearly scorable whereas 7 (11.9%) failed to give any segregation banding patterns. Among the scorable loci, 9 markers followed 1:1 segregation ratios and 43 markers 1:1:1:1 ratios (Fig. 1, Table 2). From 9 loci with 1:1 segregation ratio, alleles of each 7 SSLPs were scored and analyzed separately. Because null alleles were found, an additional designation of a, b, c or d was given to the markers (e.g. DPm132a, DPm132b). For the EST and SCAR markers, only one allele was found in each locus, the dam allele for Pmpsb and sire allele for Tag4.

Single-locus analysis

Segregation ratios that departed from the Mendelian expectation of 1:1:1:1 for two heterozygous alleles in each parent and 1:1 for an individual allele scored as presence/absence at $\alpha = 0.01$ were detected in 2 marker loci (DPm113 and DPm208). Only markers that passed the single-locus segregation test were initially used in the linkage analysis.

Map construction

For initial grouping of markers, the LOD score was set up between 3.0 and 8.0 with steps of 0.5. At the lower LOD scores, especially below 4.0, occasional spurious linkages resulted in the agglomeration of some linkage groups, while increasing LOD scores beyond 5.0 resulted in consistency of the major linkage groups.

Two-point linkage analysis using the LOD score of 5.0 resulted in 9 linkage groups (Fig. 2). All linkage groups combined markers heterozygous in the parents. Of 50, 27 marker loci were mapped with a total genome length of 103.6 cM. The maximum number of markers in a linkage group was seven in the group 1. There were cases of zero recombination between markers, (e.g. DPm107 and DPm115 in linkage group 2).

Discussion

In order to investigate gene locations in the genome of shrimp, the first genetic linkage map of *P. monodon* was constructed using AFLP markers.[8] However, the *P. monodon* genome is presumably complicated and its size has been physically estimated to be about 70% that of the human genome.[13] These results imply that the genome mapping of this species requires more DNA

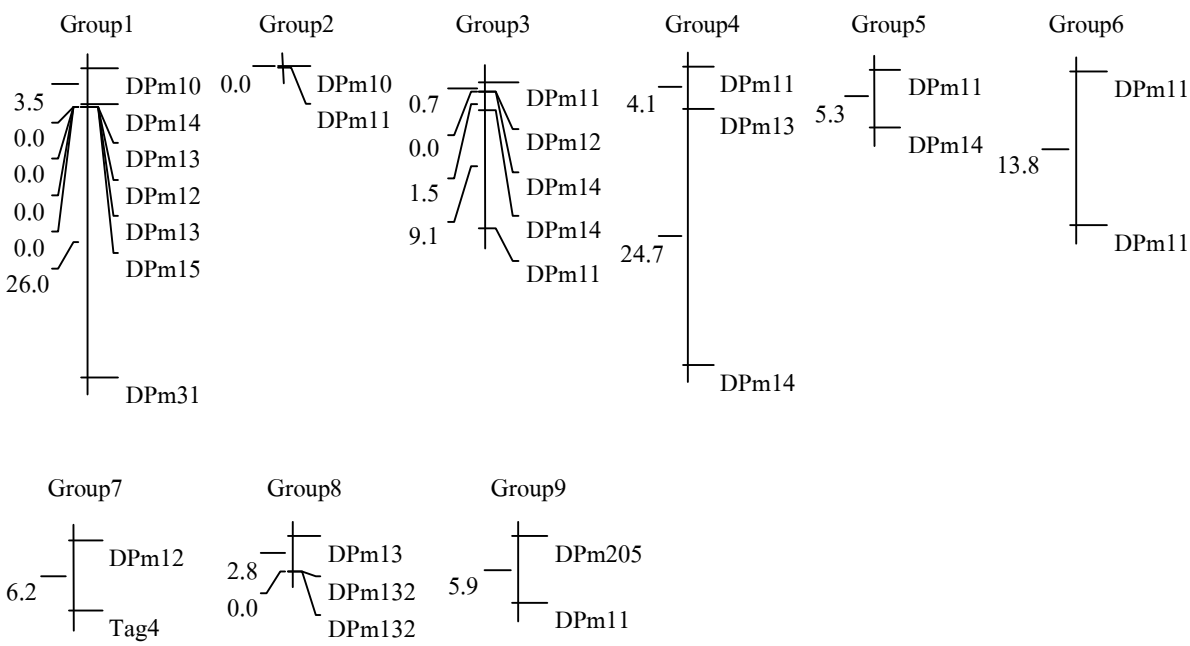

Fig 2. Linkage relationship of 27 SSLP markers in 9 linkage groups of *Penaeus monodon*.

markers in order to identify genes of interests. The linkage map for *P. monodon* that was generated in this study could be considered as the first linkage map constructed with a large number of SSLP markers.

An F1 population obtained by crossing two highly heterozygous parents, characterized for their different reproductive behavior, was studied assuming that a non inbred population would not only provide an effective strategy for limiting segregation distortion, but also give better estimates of linkage distances.[14] To a certain degree, this depends on the size of a population under study and it is only true if a large population is used. In fact, 96% of marker loci (50 out of 52) segregated according to a Mendelian ratio of 1:1 or 1:1:1:1 and only 3.9% (2 out of 52) exhibited segregation distortion (for $P<0.01$). Interestingly, there was quite a high number of SSLP loci (12/50) that had null alleles or nonamplifying alleles. In theory, null alleles are common and the flanking sequences for priming sites are possibly polymorphic. However, null alleles could be revealed either by lowering priming stringency (to accommodate sequence mismatches) or redesigning primers (to avoid polymorphic sites).[15]

In this study, we used a full-sib family derived from heterozygous parents and highly polymorphic markers, SSLPs (up to four marker alleles). However, this data can be analyzed based on the analysis of Wu et al.[10] We symbolized alleles by a, b, c, and d, when they were co-dominant with respect to the others but dominant to the null allele, symbolized by o. Simple sequence repeats, such as microsatellites, consisting of tandemly repeated multiple copies of mono-, di-, tri-, or tetranucleotide motifs, provide an ideal tool to characterize polymorphic variation in outcrossing populations.[16] To increase the resolution of genome characterization for a given outcrossing species, these highly polymorphic microsatellites should be combined with EST markers and other dominant markers to construct a single consensus map.[17,18]

In our study, the number of marker loci used was relatively low. A total of 59, instead of 102 loci developed from Wuthisuthimethavee et al.[9], were used to investigate whether SSLP markers can be mapped with a full-sib family of two- generation information. Results have demonstrated that 9 linkage groups were constructed with the total genetic distance of 103.6 cM (Fig. 2). The average space between two markers was approximately 3.8 cM. The size of linkage groups ranged from 0 to 29.5 cM, with an average of 3 SSLP loci per linkage group. There were 10 loci mapped at the same position (with zero recombination, see group 1). In fact, all markers were verified to be different clones. This event is usually observed in maps with a small number of marker loci or small population sizes. However a cluster of markers may indicate lack of recombination in the region of the chromosome in which they are found. Furthermore, linkage group 8 showed 2 linked marker loci consisting of DPm138 and DPm132 with allelic complement of the DPm132 locus (DPm132a and DPm132b) that eventually was mapped at the same location. There were 23 (46%) unlinked marker loci in this study. The high proportion of markers that were unlinked suggests the number of marker loci used.

The first genetic map of *P. monodon* was constructed using AFLP markers (Table 3)[8]. A total of 116 AFLP loci in common across three populations, 63 (54.31%) were mapped into 20 linkage groups covering a total genetic distance of 1412 cM. In our map, 9 linkage groups covered a total of 103.6 cM. Using the same genome size estimate suggested by Wilson et al.[8], the current map represented only 5.18% of the *P. monodon* genome. Therefore, the estimate in the present study could have been affected by the relatively low number of markers mapped (27 loci), however, this map can be served a good starting point for more markers to be mapped with our reference families.

Table 3. Statistics of the SSLP linkage map for *Penaeus monodon*.

Total number of markers	50
Number of markers mapped	27
Number of linkage groups	9
Number of unlinked markers	23
Total length of genome mapped (cM)[a]	103.6
Average space between two markers (cM)	3.8
Average length of linkage group (cM)	11.5
Average number of markers per linkage group	3

[a] Total length of genome mapped was calculated by Hulbert et al.[21].

The genetic linkage map will be useful for marker-assisted selection in black tiger shrimp. Additional markers are required to condense the existing map into 44 linkage groups, corresponding to the number of haploid chromosomes in black tiger shrimp.[19] High levels of microsatellite polymorphism in black tiger shrimp populations would support addition of new markers. Genotype analysis of more families and more additional marker systems, such as AFLPs[8] or ESTs[20], will improve map accuracy and increase the probability of detecting marker polymorphism.

Conclusion

Our black tiger shrimp (*P. monodon*) breeding program has been performed for selection of broodstocks producing high quality offspring, especially those with high growth rates under low salinity conditions. Previously, 102 SSLP markers were

developed for family genotyping. In this study, we initiated constructing a genetic linkage map for *P. monodon* using 57 SSLP, 1 STS, 1 SCAR markers and 76 individuals of a F1 inter-cross family. Segregating marker data was scored and analyzed with the JoinMap 2.0 program. A total of 50 marker loci confirmed to follow Mendelian segregation ratios (c^2-test at the a = 0.01 level) were grouped with a LOD score of 5.0. The results showed 9 linkage groups with 27 loci and 23 unlinked loci. This linkage map covered a total genetic distance of 103.6 cM. This genetic linkage map serves a good starting point for mapping more markers on the *P. monodon* genome with our reference families.

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