Mar. Biotechnol. 5, 311–330, 2003
DOI: 10.1007/s10126-002-0092-z

MARINE BIOTECHNOLOGY

Short Communications

High Frequency and Large Number of Polymorphic Microsatellites in Cultured Shrimp, *Penaeus (Litopenaeus) vannamei* [Crustacea:Decapoda]

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Abstract: A total of 1479 recombinant clones were obtained from a *Sau*3A-digested genomic library of *Penaeus (Litopenaeus) vannamei* and used for probe hybridization. Of the 251 clones that tested positive to one or more of the probes and were sequenced, 173 (69%) contained 573 simple sequence repeats, or microsatellites, with 3 or more repeats. The frequency of microsatellites with 3, 5, and 10 or more repeats was 1 in 0.94 kb, 1 in 2.78 kb, and 1 in 5.94 kb, respectively. To increase the number of polymorphic markers for mapping, 136 primer sets that flanked microsatellites containing single or multiple motifs with 3 or more repeats were designed and tested. Of the 136 primers, 93 (68.0%) were polymorphic in cultured shrimp, with polymorphism information content (PIC) values ranging from 0.195 to 0.873, and observed heterozygosities ranging from 10% to 100%. These markers are being used along with other markers to construct a linkage map for *P. vannamei*.

Key words: *Penaeus (Litopenaeus) vannamei*, shrimp, microsatellites.

INTRODUCTION

Marine shrimp of the superfamily Penaeoidea represent approximately a third of the world's commercially important shrimp species and account for more than 80% of the wild catch, with Pacific whiteleg shrimp, *Penaeus (Litopenaeus) vannamei* (Baldwin et al., 1998), being the leading species farmed in the Western Hemisphere. Recently, sustainability of penaeid commercial fisheries and shrimp aquaculture industry has been threatened by overfishing, habitat destruction, viral diseases, and chemical pollutants (Naylor et al., 2000). Among the viral diseases, infectious hypodermal and hematopoietic necrosis virus (IHHNV), taura syndrome virus (TSV), and white spot syndrome virus (WSSV) have caused serious economic losses to the shrimp industry worldwide (Lightner et al., 1997). When IHHNV became a serious problem in the United States, efforts were initiated to domesticate *P. vannamei* using specific pathogen-free (SPF) stocks (Lotz et al., 1995; Carr et al., 1997). When TSV emerged as a major problem for the industry, the same SPF *P. vannamei* stocks were then used for selection for TSV resistance. Although a genetic component for susceptibility of *P. vannamei* to viral diseases has been suggested (reviewed in Argue and Alcivar-Warren, 1999), basic information is lacking on the genetic loci responsible for resistance or tolerance to viruses, immune response, and high growth of shrimp. To understand these traits and to increase the rate of genetic improvement in shrimp breeding programs, the loci responsible for these traits need to be first identified through linkage and

Received August 19, 2002; accepted November 7, 2002.

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quantitative trait locus (QTL) mapping (Alcivar-Warren et al., 2002).

Simple sequence repeats, or microsatellites, are ideal markers for gene mapping owing to their variability and abundance in the genome, inheritance in a Mendelian fashion, and codominant expression (Hearne et al., 1992; Wright and Bentzen, 1994; Ozaki et al., 2000). A limited number of microsatellites have been isolated from penaeid shrimp species, including a small number from *P. vannamei* (Garcia et al., 1996; Bagshaw and Buckholt, 1997; Moore et al., 1999; Ball et al., 1998; Tassanakajon et al., 1998; Xu et al., 1999; Vonau et at., 1999; Brooker et al., 2000).

Progress in developing microsatellite-based linkage maps for *Penaeus* shrimp has been slow owing to difficulties in amplifying a large number of scorable microsatellite loci (Tassanakajon et al., 1998; Moore et al., 1999; Brooker et al., 2000). However, Xu et al. (1999) reported 99 microsatellites in *P. monodon* by direct sequencing of genomic library clones, without probe hybridization, obtained by using several vector DNA–target DNA ratios. A study was then initiated to isolate usable (polymorphic) microsatellites from a *P. vannamei* genomic library consisting of 10 different vector DNA–target DNA ratios using both direct sequencing and probe hybridization. Preliminary results obtained by direct sequencing are being reported elsewhere (Alcivar-Warren et al., 2002; Z. Xu et al., unpublished results), and the results obtained by probe hybridization are presented here.

Materials and Methods

Construction and Screening of Genomic Library

DNA was isolated from ovary of an adult *P. vannamei* (Garcia et al., 1994). The size-fractionated library was constructed following procedures performed in the laboratory of Dr. Scott Davis, Texas A&M University, College Station (Garcia and Alcivar-Warren, 1996) with minor modifications (Xu et al., 1999). Ninety micrograms of shrimp DNA was partially digested with 60 U of *Sau*3AI restriction enzyme (Gibco BRL), and 4.5 μg of vector DNA (pBluescript II SK+, Strategene) was digested with 20 U of *Bam*HI (Gibco). Digested shrimp DNA was electrophoresed on a 0.8% agarose gel in 1 × TAE (0.8 mM Tris, 0.4 mM glacial acetic acid, and 0.04 mM EDTA), and bands ranging from approximately 150 to 800 bp were eluted using Spin-X columns (Costar). DNA was precipitated using 3 M sodium acetate (pH 5.2) and 100% ethanol. The 5′ phosphate groups of shrimp DNA were removed by incubation with calf intestinal alkaline phosphatase (Promega) and 10 mM Tris-HCl at 37°C for 45 minutes. Proteins (in 100 μl mix) were degraded with 2.5 μl of 20% sodium dodecyl sulfate (SDS), 1 μl 0.5 mM EDTA, and 1 μl 10 mg/ml Proteinase K, incubated at 55°C for 30 minutes, and removed with an equal volume of phenol-chloroform. DNA was precipitated by 0.1 volume of 2 M NaCl and 2.5 volumes of 100% ETOH, washed with 70% ETOH, and then dissolved in molecular biology grade H_2O. DNA was ligated at 10 different ratios of shrimp DNA to vector DNA (Table 1) using T_4 DNA ligase (Gibco) at 15°C for 18 hours, and transformed into DH5α competent cells (Gibco), using 2 μl of the target-to-vector ligation mix. Transformed cells from each treatment were grown on 3 plates containing LB/ampicillin/IPTG/Bluo-Gal at 37°C overnight. Positive recombinant clones were picked up and streaked onto new numbered LB/ampicillin/IPTG/Bluo-Gal plates for a second screening. After overnight growth at 37°C to make sure they were positive, one half of each positive colony was picked up and grown in 3 ml of LB with ampicillin (70 mg/μl) to prepare frozen permanents and isolate DNA using an alkali lysis procedure (Garcia et al., 1996; Ausubel et al., 2002). Plasmid DNA was stored at −80°C until DNA sequencing using an ABI^{TM} 377 DNA sequencer and M13 reverse primer.

For probe hybridization, the remaining half of each white colony from plates of the second screen were streaked onto 30 plates containing numbered nylon membranes, and left overnight at 37°C. Membranes were placed first in 10% SDS for 3 minutes, followed by denaturing solution (1.5 M NaCl) for 5 minutes, neutralization solution (1.5 M NaCl, 1.5 M Tris, pH 7.5) for 5 minutes, soaked in 2× SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.2) for 5 minutes, and air dried for 10 minutes before baking them overnight at 65°C in a vacuum oven. Prehybridization washing was performed by first soaking the membranes in 2× SSC for 5 minutes and then placing them in 500 ml prehybridization washing solution (5× SSC, 0.5% SDS, 1 mM EDTA) at 50°C for 1 hour. Membranes were then placed into a Kapak/Scothpack bag with 40 ml hybridization solution (5× SSC, 0.5% SDS, 250 mM potassium phosphate buffer [0.5 M KH_2PO_4, 0.5 M K_2HPO_4, pH 6.5], 5× Denharduts) and incubated at 65°C for 1 hour. Four microliters of labeled probe was added to each bag for overnight hybridization at 37°C. Probes were labeled sequentially [$(GT)_{15}$,

Table 1. Total number of *Penaeus* (*Litopenaeus*) *vannamei* Clones Positive to Hybridization with Dinucleotide, Trinucleotide, and Tetranucleotide Probes

Treatment No. (vector-target ratio)	No. of recombinant clones	No. of clones positive to								Di, tri,- and tetra nucleotide probes[a]
		$(GT)_{15}$	$(AT)_{15}$	$(GC)_{15}$	$(CT)_{15}$	$(TAT)_{10}$	$(CTC)_{10}$	$(CTTT)_8$	$(TGTA)_8$	
1 (1:0.2)	8	0	0	0	0	0	0	0	0	0
2 (1:0.4)	12	0	0	0	0	0	0	0	0	0
3 (1:0.6)	5	0	0	0	1	0	1	0	0	1
4 (1:0.8)	7	0	0	0	0	1	0	0	0	1
5 (1:1)	80	1	1	1	4	1	0	2	1	9
6 (1:2)	133	9	2	0	4	1	3	4	1	18
7 (1:4)	191	10	4	0	5	6	5	12	8	28
8 (1:6)	345	6	4	2	16	9	5	18	6	43
9 (1:8)	189	10	9	0	8	4	5	14	4	35
10 (1:10)	509	32	13	5	43	16	34	39	24	116
Total clones	1479									251
No. of clones positive by probe hybridization[b]		68	33	8	81	38	53	89	44	
No. of clones that contain repeats of probes used, based on sequence		46	16	1	38	19	9	6	2	

[a]This column does not represent the addition of the previous 8 columns, because one clone may have been positive to more than one probe.
[b]Some of the clones that tested positive are repeated in one or more of the probes tested.

Table 2. Microsatellites Obtained from a Genomic Library of *Penaeus* (*Litopenaeus*) *vannamei* After Probe Hybridization

Microsatellite clones[a]	Forward & Reverse Primers (5′→3′)	Repeat motifs[b]	Category (Weber, 1990)	Expected size (bp)	P[e]	# of alleles	%H_o^h	GenBank Accession #
TUMXLv3.1[c]	F: TAAAACCGAAAGACAATGGCG	…**(GT)**$_3$…**(A)**$_{20}$…(TG)$_6$…(AC)$_3$…	4 Perfect, 2 compound imperfect	146	P	10	83	AF360017
	R: CTGACATTGCGTTATGATTGG	(CA)$_6$CGCTCTN(TC)$_{10}$…(TC)$_4$TN(TC)$_4$(TA)$_9$						
TUMXLv4.2	F: CATTATCGCCCTAATCACC	…**(TAA)**$_3$…**(TTA)**$_4$…	2 Perfect	251	N			AF360018
	R: GCATAATAATTGCAATAATAAC							
TUMXLv5.10[d]		…(TAACC)$_7$…(TAACC)$_3$…	2 Perfect					AF360019
TUMXLv5.16[c]	F: AATGAATCTGACCGGTTTCG	…(ATT)$_3$…**(CT)**$_3$…[f](AC)$_3$…	3 Perfect	348	P	2	100	AF360020
	R: TGGGTCGCTGGTGTGTATAG							
TUMXLv5.23	F: CCAGCCAAATTTCGTTTCC	…(TC)$_3$…**(GTCT)**$_3$**(GTCTCT)**$_{10}$**(CT)**$_{14}$**(GTCT)**$_6$…	1 Perfect, 1 compound perfect, 1 perfect	260	P	9	27	AF360021
	R: ACGGACATGTATACGCACACG	(GT)$_3$…						
TUMXLv5.27	F: CAGACCCTAAATCTCCGTGC	…**(TC)**$_4$…(CT)$_4$…(TC)$_3$…(CT)$_3$…(TC)$_5$…	5 Perfect	175	P	8	40	AF360022
	R: TGGAAAGGTCAGAGGTCACG							
TUMXLv5.35	F: CTGCTAATTGAATTTTCAGG	…**(CTTT)**$_3$…(AT)$_4$…(AT)$_3$…	3 Perfect	104	P	5	46	AF360023
	R: ACAGATAACCTAACTGACGC							
TUMXLv5.38	F: CCTTTATGACTTCCCCCGAC	…**(TC)**$_8$…**(CT)**$_4$…**(TC)**$_4$…	3 Perfect	215	P	8	84	AF360024
	R: CCGTACAGAAACGGAACGTC							
TUMXLv5.45[c]	F: TTTGTCGTTTGTCTTTCTCC	(TC)$_3$…(TC)$_4$C(CT)$_3$…(CT)$_3$…**(TA)**$_4$…**(ATCC)**$_4$…**(TC)**$_3$…	1 Perfect, 1 imperfect, 4 perfect	162	P	6	64	AF360025
	R: AGTAACTTACGTGAATGCTTGG							
TUMXLv5.54	F: TGTCTGAAGAGGGACTCGTG	…(GT)$_3$…(GT)$_6$…**(AC)**$_3$**(AT)**$_5$…**(AT)**$_{27}$…	2 Perfect, 1 compound perfect, 1 perfect	180	P	6	40	AF360026
	R: TTGTGCATTGTGGGTTTTTC							
TUMXLv5.66	F: GGGGCACTGAGACGAGTAAG	…**(CA)**$_4$**CG(CA**$_4$**T(AG)**$_3$…**(TA)**$_3$…**(AC)**$_3$…**(AC)**$_4$…	1 Compound imperfect, 4 perfect	245	P	6	36	AF360027
	R: CCGTTTTATCAGTCTCCATATACGA	(AC)$_6$…						
TUMXLv6.15[d]	F: GAGAAAACGAAATTTGGAGTC	…(TAACC)$_7$….(TAACC)$_3$…	2 Perfect	116	N			AF360030
	R: TACAGTACCTGCAATTTGGG							
TUMXLv6.23[c]	F: GTAAGCACCATAACTACCGC	…**(CCCT)**$_3$…(GT)$_{26}$	2 Perfect	210	P	6	33	AF360031
	R: GCAGAATCAGATAAAGCGAG							
TUMXLv6.31	F: CATAGACAGATATGCCTGC	…**(CT)**$_8$…**(CT)**$_9$…**(CT)**$_7$…**(CT)**$_6$…	4 Perfect	236	P	5	43	AF360032
	R: GATGACGGGGGAATGAGAG							
TUMXLv6.37	F: CGGGTTCCGCCTAGTTTTA	…**(TC)**$_3$…**(AC)**$_9$**AG(AC)**$_{31}$…(AT)$_3$…(AC)$_3$…(AT)$_3$…	1 Perfect, 1 imperfect, 3 perfect	249	N			AF360033
	R: GAGTAAGTGGGTGAGTGAGGGA							
TUMXLv6.38		…(AC)$_9$AT(AC)$_3$AT(AC)$_{20}$AT(AC)$_{33}$(AT)$_{24}$…	Compound imperfect					AF360034
TUMXLv6.46	F: CCTAGAAGGATAACGATATGCAAG	…(AT)$_3$…**(AT)**$_{19}$…**(AT)**$_{10}$…	3 Perfect	188	N			AF360035
	R: GTGGATTTCTATCATGCTGACTG							

TUMXLv6.51[c,d]		…(TAACC)$_9$	Perfect					AF360036
TUMXLv6.55[d]		…(TAACC)$_5$…(TAACC)$_3$…	2 Perfect					AF360037
TUMXLv6.57	F: CGAGTTCGGGTTAGTGATGG	…**(CA)$_4$**…**(CA)$_3$**…**(CA)$_5$**…**(CA)$_3$**…**(CA)$_{11}$CG(CA)$_{40}$**	4 Perfect, 2 imperfect	298	N			AF360038
	R: TAGTGGGTCCATGGAAGGG	…(CA)$_5$CC(CA)$_6$…						
TUMXLv6.58	F: CCCTTTACCACCTCCTTCAATC	…**(CT)$_3$**…	Perfect	166	N			AF360039
	R: AAGAGGAGGGGAAGGGTCAG							
TUMXLv6.63	F: TGTGAAGGTGTGTGAACGTG	…(AT)$_3$…[f]**(GT)$_3$**…**(GT)$_6$ATC(TG)$_8$**…**(GT)$_3$**…**(GT)$_4$**…	2 Perfect, 1 imperfect, 4 perfect	186	P	4	17	AF360040
	R: CTGCAACCTTTGGTCTTGC	(TG)$_3$…(TA)$_3$…						
TUMXLv6.98[c]	F: TGCGTATTAGTACAATGGATGG	(TC)$_3$…(CA)$_{43}$TA(CA)$_{21}$TA(CA)$_{30}$…**(TA)$_3$**…	1 Perfect, 1 imperfect, 1 perfect	252	P	3	50	AF360040
	R: TGCAAAGGTGGCATAAAAG							
TUMXLv6.116[c]	F: ATGAAAAACTCGCACACTTC	…(GT)$_4$…(GT)$_6$…**(ATT)$_5$**…**(AAT)$_3$G(ATA)$_2$(AT)$_5$**…	3 Perfect, 1 compound imperfect, 4 perfect	238	P	9	10	AF360028
	R: TATCGTCACCATAACCAGAG	**(CA)$_3$**…**(TA)$_3$**…**(TC)$_8$**…(GAT)$_5$…						
TUMXLv6.124	F: GAAGTGCTTCAGTTGGCGAC	…**(AC)$_3$**…**(AG)$_3$**…(TC)$_7$…	3 Perfect	168	P	2	33	AF360029
	R: CCGGATATCTGTTGCGTTTC							
TUMXLv7.14[c]		…(CA)$_{29}$CG(CA)$_{12}$T(AC)$_3$GCAC(AT)$_3$ACATAC(AT)$_{36}$N TATGT(AT)$_3$…(GT)$_3$NT(GT)$_{11}$GC(GT)$_{27}$	1 Compound imperfect, 1 imperfect					AF360049
TUMXLv7.19[d]		…(TAACC)$_9$…(TAACC)$_3$…	2 Perfect					AF360053
TUMXLv7.31[g]	F: GAACCTGCGTTGAGGCTACC	…**(CA)$_{38}$AA(CA)$_6$(TA)$_3$TT(TA)$_{34}$**…**(TG)$_3$**…**(TA)$_3$**…	Compound imperfect, 2 perfect	350	P	2	14	AF360054
	R: GAAACACAGAGCAGAGAAAAACG							
TUMXLv7.56	F: CCATGGCTTTCCTCTTCTTTC	…**(TCC)$_5$**…**(CCT)$_3$**…**(CCT)$_3$**…**(TC)$_4$**…**(TC)$_4$**…**(TC)$_3$**…	10 Perfect	327	P	4	75	AF360055
	R: AGGTAGGGAAGTCGTGAGGG	**(TC)$_3$**…**(TC)$_3$**…**(CT)$_3$**…**(CCT)$_3$**…						
TUMXLV7.74	F: CCTGCGCAATACTGGATATG	…**(AT)$_3$**…**(AT)$_3$**…(AC)$_7$…	3 Perfect	214	P	4	33	AF360056
	R: CGAGGTGTAGTTGTGCTTTGG							
TUMXLv7.97[c]	F: TGTCGTTAGTGCAGCTCATTC	…(AT)$_5$…**(TTC)$_3$TT(TTC)$_3$TT(TTC)$_3$**…(TCC)$_3$C(TCC)$_5$	1 Perfect, 2 imperfect	176	P	3	33	AF360057
	R: GGGGAGGAATAAGAGGAAAGG							
TUMXLv7.102	F: TTTGGGAAGTAAGGCTGGAG	…**(CCT)$_4$**…**(CTT)$_3$(TC)$_3$**…**(CCT)$_3$**…**(TCT)$_3$**…**(CTT)$_3$**…	1 Perfect, 1 compound perfect, 4 perfect, 1 compound imperfect, 5 perfect	320	P	2	58	AF360042
	R: GGAGTAGACGGTTAAGGAGCAG	**(TCC)$_3$**…**(CT)$_5$TT(CTC)$_4$**…(CG)$_3$…(AAC)$_3$ ..(ATA)$_3$…(AAT)$_3$…(AAT)$_3$…						
TUMXLv7.121[c]	F: GGCACACTGTTTAGTCCTCG	(GTT)$_3$…(GA)$_3$…(TC)$_3$…**(GT)$_3$**…**(TC)$_3$**…**(TC)$_3$**…**(TC)$_3$**…	7 Perfect	242	P	3	42	AF360043
	R: CGAACAGAATGGCAGAGGAG							
TUMXLv7.124[c,d]		…(TAACC)$_4$…(TAACC)$_3$	2 Perfect					AF360044
TUMXLv7.127	F: GAATGGGAGGAGAAGGATAG	…**(AAC)$_3$(AG)$_3$**…(T)$_{26}$AT(TC)$_4$…(TC)$_3$…(TACA)$_3$	1 Compound perfect, 1 imperfect, 3 perfect, 1 compound perfect, 1 perfect	105	M	1		AF360045
	R: TTCCACGTGGTTTCCCGATG	…(AT)$_4$…(AT)$_3$(CT)$_5$…(AT)$_3$…						

Continued

Table 2. Continued

Microsatellite clones[a]	Forward & Reverse Primers (5′→3′)	Repeat motifs[b]	Category (Weber, 1990)	Expected size (bp)	P[e]	# of alleles	%H_o^h	GenBank Accession #
TUMXLv7.132	F: ATCCATCCATTTTTCCCCTC R: CTTTGGAGGGACTGGGAGA	…$(CT)_3$…**$(CT)_4$**…	2 Perfect	193	P	5	50	AF360046
TUMXLv7.134[c]		…$(GA)_{20}$…	Perfect					AF360047
TUMXLv7.138	F: AGACACATACAGACGCACGC R: GAGTTGCTCCCAAACGCTAC	…**$(CA)_6TAG(AC)_3GC(AC)_3$**…**$(AC)_3$**…**$(CA)_{19}$**…**$(CA)_7$**…	1 Imperfect, 3 perfect	329	P	3	67	AF360048
TUMXLv7.146[c]	F: AACCTAGACATTTAGAAGGCACA R: GTTTGAATTTCTAAGGCTACAGAAGA	…**$(TA)_3$**…$(AT)_{20}$	2 Perfect	102	M	1		AF360050
TUMXLv7.148	F: CATCGCTAAAATTCCGAAGC R: TAAAAATGAGGGGGTTGGAG	…$(AT)_3$…$(AG)_5$…**$(CT)_{11}$**…**$(TA)_5$**…	4 Perfect	273	P	4	56	AF360051
TUMXLv7.167	F: ACACACCCATCCAACTACCC R: GGCCTATGGTTTGTCTGAGG	…**$(CA)_3TA(CA)_3AG(CA)_{35}AAC(ACGC)_4$**…**$(CA)_3A$** **$(ACCC)_3(AC)_3$**…**$(ACCC)_3(AC)_6$**…**$(AC)_7(GC)_3$**…	1 Compound imperfect, 1 imperfect, 2, Compound perfect	321	P	4	42	AF360052
TUMXLv8.2[c]	F: CCTCCTGTCCATTCAGCAG R GGTCAGATATGTATTCGAGTRCGG	…$(AT)_3$…**$(AT)_3$**…**$(AT)_3$**…**$(AT)_3$**…**$(AT)_3$**…**$(AC)_3$**…**$(AT)_3$** …**$(AT)_3$**…**$(AT)_3$**…$(AT)_3$	10 Perfect	244	P	9	83	AF360070
TUMXLv8.9	F: CCCTTTCGATTGAAACCAAC R: GGAGTTGCGCTAAGTTAATTCC	…$(AT)_3$GTG$(TA)_3$…$(GAA)_4$…$(AAT)_3$…$(TC)_3$…**$(TAA)_3$**… **$(TAA)_3$**…**$(AAT)_3GAC(AAT)_3T(ATA)_3$**…**$(AAT)_3$**…$(TC)_3$… …	1 Imperfect, 5 perfect, 1 imperfect, 2 perfect	251	P	4	42	AF360089
TUMXLv8.11[d]		…$(TAACC)_9$…$(TAACC)_3$… $(TAACC)_6$…$(TAACC)_3$…$(TAACC)_7$…$(TAACC)_3$…	6 Perfect					AF360058
TUMXLv8.25	F: ATTCTTTGTGTTTCTTCGCC R: CGTCCCTGAAACTTTATCTCC	…**$(CA)_{3...}$**[f]**$(GT)_5$**…**$(GT)_3$**…	3 Perfect	113	P	2	80	AF360075
TUMXLv8.32	F: TTACCGCCTAAGAGCGAATG R: TGTCCTTTCGTACCAGTCAAG	…**$(CA)_3$**…**$(TA)_4$**…	2 Perfect	220	P	8	67	AF360080
TUMXLv8.37[d]		…$(TAACC)_4(TAACA)_3$…$(TAACC)_3$…	1 Compound perfect, 1 perfect					AF360085
TUMXLv8.67[c]	F: AGAGTCCTTGGTGAGTAGC R: GAGCGATAGAGTGCAATAAAG	…**$(TC)_3ACC(CT)_3$**…**$(CT)_3TTA(TC)_3$**…**$(TC)_4$**…**$(TC)_3$**… **$(TC)_4$**…**$(TC)_6$**…[F]**$(TC)_4$**…$(TC)_3$…$(TC)_{11}$TTTTCTATA$(TC)_{11}$	2 Imperfect, 6 perfect, 1 imperfect	353	N			AF360086
TUMXLv8.77[c]	F: TGCGAGTGCTATGAATACTG R: ATGTATAAATGTCAGCGCAC	…$(AC)_5$…$(AC)_5(AT)_4GA(GT)_4ATT(TA)_3(TG)_3CG(TG)_3$… **$(CA)_3$**…**$(AC)_3GTG(TA)_3$**…**$(TG)_3GAAATATG(TA)_3$**… **$(TA)_4TG(TA)_4$**…$(AT)_3AC(at)_3gc(at)_{23}$… $(TA)_3CATATTCATATTGATATGN(AT)_4GC(GT)_3$… $(AC)_3$	1 Perfect, 1 compound imperfect, 1 perfect, 2 compound imperfect, 2 imperfect, 1 compound imperfect, 1 perfect	186	N			AF360087

TUMXLv8.79[d]		…(TAACC)$_6$…(TAACC)$_3$…	2 Perfect					AF360088
TUMXLv8.123	F: TTTGCACCAGTTCTGAAAGG R: CTATGCATCCCATTTGTAACG	…**(AG)$_3$(AAT)$_6$(TAT)$_5$C(ATT)$_4$ATC(ATT)$_4$(ATA)$_{14}$…** (CA)$_3$…	1 Compound imperfect, 1 perfect	268	P	6	25	AF360059
TUMXLv8.127[c]		…(CT)$_6$(CTTT)$_3$TTTCTCCCTCAA(TC)$_{17}$TT (TA)$_{15}$TN(TG)$_{24}$	Compound imperfect					AF360060
TUMXLv8.149[c]		…(CT)$_{49}$	Perfect					AF360061
TUMXLv8.173[c]		…(CTTT)$_3${[(T)$_3$AATCGTTCG(T)$_4$(CTTT)$_3$]$_2$TCAATCG TTCG(T$_4$)(CTTT)$_4$}$_2$…(CTTT)$_3$…	1 Compound imperfect, 1 perfect					AF360062
TUMXLv8.176	F: GCAACGCAATATAGCTC R: TCAAGGGAACAAAGTCAAG	…**(AT)$_3$**…**(GT)$_3$**…	2 Perfect	168	P	3	50	AF360063
TUMXLv8.177	F: TCTCTTAAGGTACCCCTG R: CAAGAACAAGAAAGAGAAGG	…(TC)$_4$…[f]**(TCT)$_3$**…**(CTT)$_3$**…(CCT)$_4$CA(TTC)$_9$CTCTTCT (TCC)$_3$…(CCT)$_4$…(TCC)$_4$…	3 Perfect, 1 imperfect, 2 perfect	144	M	1		AF360064
TUMXLv8.179	F: CGCATTTCAAGTGCTCAAGG R: TCATGCGCTATTGTGGACAG	…(TGG)$_3$…(AT)$_4$…**(TTTTA)$_3$**…**(TAT)$_6$**…	4 Perfect	211	P	3	90	AF360065
TUMXLv8.182[d]		…(TAACC)$_8$…(TAACC)$_3$…	2 Perfect					AF360066
TUMXLv8.184	F: TTCAAACTACGACCAGAGC R: TGTAAATATCACACGCGG	…**(TA)$_4$**…**(CA)$_3$CT(CA)$_4$TG(CA)$_{16}$A(AC)$_3$(AT)$_3$**…	1 Perfect, 1 compound imperfect	250	P	6	50	AF360067
TUMXLv8.190[c]	F: TCAAACGTTCATGTTCTGAC R: GATACCGTTCGTACAAAGAAG	…**(AC)$_3$**…(TC)$_{24}$	2 Perfect	60	M	1		AF360068
TUMXLv8.193	F: GATGTACACAACTGTACTTCG R: GAGATGATAAGAGAACGAAAG	…**(AT)$_3$**…	Perfect	169	P	9	86	AF360069
TUMXLv8.216	F: GCTATCCTCATCCTCATTAC R: GATGGTGTTATCAATGGC	…**(ATT)$_5$ATCATTATAC(TTA)$_3$AA(ATT)$_3$**… **(ATT)$_3$AC(TAT)$_3$…(ATT)$_3$…**	2 Imperfect, 1 perfect	300	P	9	75	AF360071
TUMXLv8.220	F: GATGTGGTTGATGAAGTGATG R: CGCATTATCTAAATGGCAAG	…**(TA)$_4$TG(TA)$_3$(CA)$_3$(CGCA)$_3$(CTCA)$_6$**… **(CA)$_3$(CGCA)$_3$(CA)$_{33}$TT(TA)$_5$CA(TA)$_3$CA(TA)$_3$**…	2 Compound imperfect	261	P	11	75	AF360072
TUMXLv8.224	F: TCGTGCGGTGAAATATAGGC R: TGAATGTCCCGTTGATTGAC	…(CAC)$_3$…(CT)$_3$…**(AT)$_4$**…**(AC)$_3$**…**(TG)$_3$**…	5 Perfect	300	P	2	73	AF360073
TUMXLv8.244	F: GAGACGGCACCAAAATTAGTC R: TTCATATGTCGTCCTCGCTG	…(AT)$_3$…**(TC)$_5$**…**(TC)$_6$**…**(TC)$_{15}$(TA)$_{25}$**…(TC)$_7$…	3 Perfect, 1 compound perfect, 1 perfect	295	P	5	57	AF360074
TUMXLv8.256	F: GGACTCACACTTCTGGTTC R: GGCTGCACCTTGTAAGTC	…**(AAT)$_4$**…	Perfect	166	P	6	67	AF360076
TUMXLv8.276[c]	F: TATCTTCTTCAACTCGATGG R: GAATAACAGACATGACACTGAC	…(AT)$_3$…(AT)$_3$…**(GTG)$_3$**…(TC)$_6$…(CT)$_3$TTT(TC)$_3$T(TC)$_{31}$	4 Perfect, 1 imperfect	70	N			AF360077
TUMXLv8.278[d]		…(TAACC)$_4$…(TAACC)$_3$…(TAACC)$_7$…(TAACC)$_3$…	4 Perfect					AF360078
TUMXLv8.307		…(CT)$_3$…(TC)$_5$…(TC)$_3$…(CT)$_3$…(TC)$_3$…	5 Perfect					AF360079
TUMXLv8.324	F: TCAGTATTATTAACATCGTCACTGTTC R: TGGTGATAATGAAAAAGATGGTG	…**(TAT)$_5$CAC(TAT)$_3$**…**(CAT)$_3$**…**(TAT)$_3$**…	1 Imperfect, 2 perfect	199	P	4	60	AF360081

Continued

Table 2. Continued

Microsatellite clones[a]	Forward & Reverse Primers (5′→3′)	Repeat motifs[b]	Category (Weber, 1990)	Expected size (bp)	P[e]	# of alleles	%H_o^h	GenBank Accession #
TUMXLv8.327	F: GGTAAGTGGTCATCACGC	…**(GC)**$_4$(ACGC)$_4$…**(CACT)$_3$TAT(ACTC)$_{21}$(AC)$_{39}$**…(CG)$_3$…	1 Compound perfect, 1 compound imperfect, 1 perfect	279	P	8	36	AF360082
	R: GAGCTGGTGATGGATGAG	…						
TUMXLv8.332	F: GTCTGGATGAATGGAGAGCG	**(CT)**$_3$…**(CT)**$_6$…**(TC)**$_3$…**(CTTT)**$_3$…**(TC)**$_3$…	8 Perfect	272	P	6	50	AF360083
	R: ATAAAAGAGAAAGAGAGAG	**(CTCTTT)**$_3$…**(CTCTTT)**$_8$…[f]**(TC)**$_9$…						
TUMXLv8.342[c]		…(AT)$_{28}$	Perfect					AF360084
TUMXLv9.6		…(AC)$_7$TC(AC)$_{11}$AA(AC)$_{42}$TC(AC)$_3$TC(AC)$_{10}$…	Imperfect					AF360110
TUMXLv9.8		…(GA)$_3$…	Perfect					AF360113
TUMXLv9.18[c]		…(TC)$_3$…(TG)$_4$TC(TG)$_7$CG(TG)$_{15}$	1 Perfect, 1 imperfect					AF360106
TUMXLv9.28	F: CTTCACCTCCCCTCTCACAC	…(CCT)$_3$…**(ATC)**$_3$…**(CT)**$_3$…**(CTTTT)$_3$TTT(CTT)**$_3$…	3 Perfect 1 compound imperfect	155	P	6	46	AF360108
	R: CAATCATCACCGGTCCTACC							
TUMXLv9.43[c]	F: GAGAGCAAATAAGAAAGGGC	…(TG)$_4$C(GT)$_4$TC(GT)$_6$(GC)$_3$…(GT)$_3$TTA(TG)$_4$…(GGA)$_3$	1 Compound imperfect, 1 imperfect, 2 perfect, 1 imperfect, 2 perfect	219	P	11	75	AF360109
	R: AGGATGCAAATGATAACGAG	[f]**(GA)**$_3$…**(CT)$_3$CC(CT)$_3$CC(CCCT)$_6$(CT)$_{10}$**…(TC)$_6$…(TC)$_3$						
TUMXLv9.60	F: GGCCAAGTTTACAATGAC	…(CA)$_3$…(TA)$_4$…(CA)$_3$…(CT)$_3$…(CA)$_3$…**(AC)**$_6$…**(CA)**$_4$T	6 Perfect, 1 imperfect, 2 perfect	300	P	2	25	AF360111
	R: GCAAGGTATGTTTAGTG	**AT**						
		(AC)$_6$AAT(CA)$_3$T(AC)$_5$ATT(CA)$_3$T(AC)$_6$T(CA)$_4$TAT						
		(AC)$_5$AAT(CA)$_3$…(AAT)$_3$…(TAAA)$_3$…						
TUMXLv9.77	F: CCATTGATGTTGCATCTGAGC	…(AG)$_4$…(AT)$_3$…**(ATA)**$_3$…	3 Perfect	206	P	7	67	AF360112
	R: CGGAAGAAGCAACTACGAGG							
TUMXLv9.90	F: GACCAAAGGATATTGGCTCG	…**(GA)**$_3$…**(GA)**$_3$…**(GA)**$_9$…	3 Perfect	295	P	5	64	AF360114
	R: GTAATCAGGAGATGGTCCGC							
TUMXLv9.93	F: CACCACCGAAAAGGTAGGAG	…**(TG)$_5$ATG(GT)$_4$…(AG)$_9$**…	1 Imperfect, 1 perfect	283	P	7	50	AF360115
	R: TGGGAGAGGTTAGTCATGGG							
TUMXLv9.99[d]	F: CGAAATTTGGAGTCTCATGG	…**(TAACC)**$_{10}$…**(TAACC)**$_3$…	2 Perfect	127	N			AF360116
	R: GGTTACAGTACCTGCAATTTGG							
TUMXLv9.103	F: CACCAAAACGAACGAAACG	…**(GA)**$_3$…**(TC)**$_{11}$…	2 Perfect	216	P	4	75	AF360090
	R: GGATAAAAACGAATTGTATACCG							
TUMXLv9.105		…(CA)$_6$AA(CA)$_3$AA(CA)$_{55}$…(AT)$_3$…(AT)$_3$…	1 Imperfect, 2 perfect					AF360091
TUMXLv9.107[c,d]		…(TAACC)$_4$…(TAACC)$_3$…	2 Perfect					AF360092
TUMXLv9.113[c]		…(TG)$_{16}$	Perfect					AF360093
TUMXLv9.116	F: GATGACCTGCCTTTCTCTGC	…**(TTC)**$_3$…**(CTT)**$_3$…**(CT)**$_3$…**(CTT)**$_3$…(GT)$_3$T(TCC)$_3$…	4 Perfect, 1 compound imperfect, 2 perfect	146	P	7	67	AF360094
	R: GGGAGAGATGATGGGAAGAAG	(TCC)$_3$…(CT)$_3$…						

TUMXLv9.117	F: CATCTGTTTAACACGGCAAC R: GACCCTGCAATTTTTAGTGA	…**(AT)**$_4$…**(TA)**$_5$…	2 Perfect	216	N			AF360095
TUMXLv9.136	F: CAAGCCATAGGGAAGAATCTG R: CGCGTTTCTTGGTTCCTTA	…**(TA)**$_3$…**(TA)**$_3$…**(TA)$_{23}$CA(TA)$_3$…**	2 Perfect, 1 imperfect	213	P	6	57	AF360096
TUMXLv9.144	F: CCAAAATCATCACCATTACC R: TTGACAACGATTACCTTCAC	…(TCC)$_6$…(ATC)$_8$…(ATT)$_4$ACC(ATTATC)$_3$… (ATTATC)$_3$…(TCA)$_5$C(CAT)$_3$…(TGA)$_3$TAA(TGA)$_3$… (TAA)$_3$…(ATA)$_4$…(ATT)$_6$ACC(ATT)$_9$…(TTA)$_3$… **(CAT)$_7$AAT(CAC)$_4$**…	2 Perfect, 1 compound imperfect, 1 perfect, 2 imperfect, 2 perfect, 1 imperfect, 1 perfect, 1 compound imperfect	144	P	11	80	AF360097
TUMXLv9.145	F: GAGAAGAGGCTGCTTTGTCG R: TGACTTTGAACTGGTGTGCG	…**(TA)$_3$CAA(AT)$_3$**…	Imperfect	278	P	2	67	AF360098
TUMXLv9.149	F: CTGGGAATATCCCATGAAGG R: GACAGCAGAGAAACTGACTGC	…**(TA)**$_3$…**(TA)**$_3$…	2 Perfect	249	P	3	78	AF360099
TUMXLv9.151	F: CATTGTTAGCATGATTGCACAG R: CGTAAAACAAATCGAAATGGG	…**(AT)**$_3$…(CT)$_4$…	2 Perfect	199	M	1		AF360100
TUMXLv9.158	F: CAATGCCTGTTAAGCAAAC R: CGCTATTTAAGCGAGTGTG	…(CA)$_3$…**(GT)$_3$ATGTAG(GT)$_6$…(TC)$_5$…** **(TC)$_{18}$TTT(TATC)$_3$…**[f]**(AC)$_4$**…	1 Perfect, 1 imperfect, 1 perfect, 1 compound imperfect, 1 perfect	245	N			AF360101
TUMXLv9.161	F: GCGAGAGAACACATGACC R: CGGAATGTAATATTTCCCAC	…**(CA)$_4$CGCACG(CA)$_{19}$CG(CA)$_9$CG(CA)$_5$CG(CA)$_{57}$** **(CGCA)$_3$(CA)$_2$CG(CA)$_{11}$**…(TA)$_{29}$CA(TA)$_2$CA(TA)$_5$…	2 Imperfect	292	P	4	67	AF360102
TUMXLv9.173	F: TCATACACCTGGACTTATGC R: CCTTTCCACACTTTAAGAGG	…(GAA)$_3$…**(CTC)**$_5$…**(CCT)**$_3$…**(CCT)**$_3$…	4 Perfect	162	N			AF360103
TUMXLv9.174	F: CACATCATGTCACTGCTACGAC R: GCTGCACAATCAACTTGCTTAC	…**(AT)**$_3$…	Perfect	234	P	2	100	AF360104
TUMXLv9.178	F: CATTGAAAACGGAATCCTCG R: GATATTCCCATCAACACAGCG	…**(GC)**$_3$…**(CT)**$_5$…	2 Perfect	196	P	3	75	AF360105
TUMXLv9.182	F: AGTATCATTATCATTGCCGC R TTACTGAACCGTACTGATTGC	…(ATT)$_3$…(TG)$_3$…**(ATT)**$_4$…**(TTA)**$_3$…(CTA)$_3$(ATG)$_3$… (ATA)$_3$TAATAC(CTA)$_3$…	4 Perfect, 1 compound perfect, 1 compound imperfect	176	P	4	50	AF360107
TUMXLv10.2	F: TCCAAACTGTCCAATAAACC R TTTAATATTGCACCCCTCC	…**(GAG)**$_3$…(TA)$_3$…(GT)$_3$…(AT)$_3$…	4 Perfect	184	N			AF359957
TUMXLv10.7[c]	F: GTGTAATCCTTCCGCTTTCG R: TGCATGTGTAATGATGCTGAAG	…**(AT)**$_3$…**(CA)**$_3$…(AT)$_3$(GT)$_{18}$	2 Perfect, 1 compound perfect	222	M	1		AF360014
TUMXLv10.14	F: CAGTCTACACGCACAGGCAC R: TTATACGGCGGTTCTCTTGG	…**(CA)**$_{35}$…**(CA)**$_3$…(CCG)$_3$…	3 Perfect	260	P	4	57	AF359947
TUMXLv10.27	F: GATCGGAAAGGACCGATAC R: CAATGGGAATTTCGCAAGAC	…**(AC)**$_4$…**(AC)**$_7$…**(TA)$_3$ATTACAA(AC)$_3$AT(AC)$_3$AAG** **(CA)$_4$TA(CA)$_3$TACACG(TA)$_3$**…	2 Perfect, 1 imperfect	265	P	3	20	AF359979

Continued

Table 2. Continued

Microsatellite clones[a]	Forward & Reverse Primers (5′→3′)	Repeat motifs[b]	Category (Weber, 1990)	Expected size (bp)	P[e]	# of alleles	%H_o^h	GenBank Accession #
TUMXLv10.28[c]		…$(ATT)_3$G$(TTA)_3$GCG(TCATTATTA)$_3$…$(TTA)_4$… $(ATT)_3$…$(ATT)_4$…$(ATT)_4$ACTATTACC$(ATT)_4$…$(TTA)_3$	1 Compound imperfect, 3 perfect, 1 imperfect, 1 perfect					AF359981
TUMXLv10.33	F: CGAAGAGATTTATCCAGGG R:CGTGCATTATTATCCTTTCC	…$(TTC)_3$…**(ATA)$_3$**…**(AAT)$_3$**…**(AAT)$_4$**…$(GA)_3$…$(ATC)_{3…}$	6 Perfect	257	P	7	55	AF359992
TUMXLv10.41	F: CTGCTATTGTTATCTTGTCAC R:CTATGATAACGATAGTCATG	…**(TTG)$_3$**…$(AG)_{43}$…$(GA)_3$…	3 Perfect	198	M	1		AF360003
TUMXLv10.44	F: ATGTCCCATTCCCAATTCAG R:AGTCGCTATTTGTCGCGCCT	…**(CT)$_{44}$**…$(TG)_3$G$(GT)_3$…$(GT)_4$…$(AC)_4$…$(GA)_5$…	1 Perfect, 1 imperfect, 3 perfect	142	P	4	0	AF360005
TUMXLv10.47[c]		…$(AT)_4$AC$(AT)_{16}$	Imperfect					AF360008
TUMXLv10.55[c]		…$(ATT)_3$C$(TAT)_3$…$(AG)_{34}$	1 Imperfect, 1 perfect					AF360011
TUMXLv10.62	F: CTCGTAAACATCGCAAAAC R: AGGAAGGAAGAAAAATAGGG	…$(AT)_5$…$(CT)_3$TT$(GT)_3$…**(CTTT)$_3$**…**(GT)$_3$**…**(CTTT)$_4$**… **(GT)$_3$**…**(CTTT)$_3$(GT)$_4$**…$(CTTT)_5$…	1 Perfect, 1 compound imperfect, 4 perfect, 1 compound perfect, 1 perfect	219	P	4	100	AF360012
TUMXLv10.68	F: GCAGTACATCTGCATCCTTC R: ATGAGGAAAGCCAAAAGG	…**(AAAG)$_3$**…	Perfect	88	N			AF360013
TUMXLv10.93	F: GACGAACAGCCAGTCAACC R: GGGGATAGGGTAGCGGAAG	…$(AT)_3$…**(TC)$_4$**…**(TC)$_4$**…**(TC)$_{14}$**…**(TC)$_3$**…	5 Perfect	288	P	4	63	AF360015
TUMXLv10.96	F: GAATACGTGGGGATGCGTAG R: AGGTGGCAATAACGTGGAAG	…**(GC)$_4$TAA(TG)$_4$**…**(GT)$_3$AT(GT)$_2$AT(GT)$_3$**…	1 Compound imperfect, 1 imperfect	238	P	2	90	AF360016
TUMXLv10.117	F: CTCCAGGACCGATAATGAGG R: CGACAGTCAAAACAAACATCC	…**(TC)$_3$**…**(TCG)$_3$**…$(TC)_{25}$	3 Perfect	118	P	4	50	AF359944
TUMXLv10.121	F: TAGTATGCATATTGATGATT R: CCTATAAAAACCTANCCTA	…**(GT)$_7$T(TGAG),**…	Compound imperfect	98	P	2	100	AF359945
TUMXLv10.127		…$(AT)_{17}$…$(ATAG)_3$	2 Perfect					AF359946
TUMXLv10.141	F: CTACTTATCGGTCTTTCTACTTACC R: CTTAGTGTTTTGTTCACCCC	…**(AT)$_3$**…**(TG)$_4$(CG)$_3$**…**(AC)$_7$(ACGC)$_3$GC(AC)$_{28}$**… $(TGG)_3$…$(AG)_3$…$(GT)_3$…	1 Perfect, 1 compound perfect, 1 imperfect, 3 perfect	206	N			AF359948
TUMXLv10.146	F: GTCGAGGCAAAGAGAAGTAG R: TGTGAGAGTGAAAGTGTTGG	…$(TG)_3$…**(CTC)$_3$**…$(AC)_3$…	3 Perfect	122	P	5	83	AF359949
TUMXLv10.147	F: CTATCCTTTCCACCTCCTTC R: GACCTGGAGGAGAATAGAGC	…**(TC)$_3$**…**(TC)$_3$**…**(TTC)$_3$C(TTC)$_2$CTT(TC)$_3$**…**(TC)$_4$**…	2 Perfect, 1 compound imperfect, 1 perfect	194	P	3	58	AF359950

TUMXLv10.150[c]		…$(TG)_{34}$…	Perfect					AF359951
TUMXLv10.176	F: TTGCGTTTCTGTCCTTTATG	…$(TC)_3$…**$(GT)_4$**…**$(TTC)_3$**…$(TA)_4$…$(CA)_6$TCTATC$(TA)_4$…	4 Perfect, 1 compound imperfect,	240	P	2	25	AF359952
	R: AGAAATGGAGAACGCAGTAG	$(TG)_4(AC)_{46}$…	1 compound perfect					
TUMXLv10.177[c]	F: GATCGTACCCCGTGACCC	…**$(ACCCCC)_3$**…$(GT)_3$…$(ATAC)_3$	3 Perfect	73	P	2	33	AF359953
	R: TCAAATACACCCCCAACACA							
TUMXLv10.182[c]		…$(CA)_6$TAA$(AC)_3$AA$(AC)_3$…$(TA)_{19}$…	1 Imperfect, 1 perfect, 1 imperfect,					AF359954
		$(AC)_{38}$NC$(AC)_9$NC$(AC)_{18}$…$(AT)_{52}$	1 perfect					
TUMXLv10.186		…$(CT)_3$…	Perfect					AF359955
TUMXLv10.191[c]	F: CTCCTTCTCCCTCTCCCACT	…$(TTA)_3$…**$(TC)_3$**…	2 Perfect	207	P	3	50	AF359956
	R: TATCTCGCCATCAGGAGACC							
TUMXLv10.200	F: GCAACAGACATAATGTAGGC	…**$(GC)_3$**…	Perfect	105	M	1		AF359958
	R: AATGCTCGTGCCCTCATC							
TUMXLv10.201	F: ATGCTGCATCATCTCTGAC	…**$(CRC)_6$TTTCTCT$(TCC)_5$**…**$(TTC)_3$$(CTC)_3$**…$(CTTT)_3$…	1 Imperfect, 1 compound perfect,	163	P	6	20	AF359959
	R: AGGAAAAGACGGTGAAATAG	$(TC)_3(CTT)_3$…	1 perfect, 1 compound perfect					
TUMXLv10.204	F: ACACTTTACAAGGACCATCG	…**$(TTA)_3$**…[f]**$(GT)_3$**…	2 Perfect	216	P	3	83	AF359960
	R: CACCAACGAAAAGAACTACAG							
TUMXLv10.205	F: GAAGTTACCCAATGTTCGCC	…**$(AC)_3$**…**$(CA)_3$**…**$(GA)_3$**…	3 Perfect	217	M	1		AF359961
	R: TGGAGAAATGCRGTGGTCTG							
TUMXLv10.206[c]		…$(ATT)_3$…$(TC)_3$…	2 Perfect					AF359962
TUMXLv10.207	F: GATCACTAGCCATATTTCATCC	…**$(TC)_3$**…$(TC)_6$…	2 Perfect	97	P	2	67	AF359963
	R: ATCGCATAATGAGCAAACTGG							
TUMXLv10.208	F: TGGAGGTCGAGAGGACAC	…**$(CGC)_4$**…**$(GGT)_3$**…	2 Perfect	121	P	2	36	AF359964
	R: GTTGGCATCTCAAGAAAAC							
TUMXLv10.209	F: GGTCTATTGCAGCTGATATC	…**$(AT)_6$**NT$(AT)_4$NT$(AT)_{12}$A$(GT)_3$…	Compound imperfect	151	N			AF359965
	R: GGCTTAGAATGGGCAGGT							
TUMXLv10.213[d]		…$(TAACC)_7$…$(TAACC)_3$…	2 Perfect					AF359966
TUMXLv10.216	F: ATTGTTGCATTTITCCAGGC	…**$(CT)_3$**…	Perfect	243	N			AF359967
	R: CAACTCAAACGAACAGCCAC							
TUMXLv10.218	F: GATCATTAATATACCAAGTAGTG	…**$(TA)_3$**…**$(TA)_3$**…**$(AG)_3$**…	3 Perfect	299	P	3	50	AF359968
	R: GGACCTCTTTATGGAGACGG							
TUMXLv10.220	F: CGGAGTAAGGTACGGACTG	…**$(GCG)_4$**…	Perfect	163	P	3	55	AF359969
	R: GTGGAGGTCGAGAGGACAC							
TUMXLV10.221	F: CTCTCGTTGGCTAAATGAAC	…**$(TC)_3$**…**$(CT)_3$**…**$(TTA)_3$**…$(AG)_3$…	4 Perfect	217	N			AF359970
	R: GTAGCAAACAGCAATTAGGG							
TUMXLV10.223	F: CGTTACTTTCATCCTTATCC	…**$(TAT)_4$$(CAT)_3$$(TAT)_3$**…**$(ATT)_3$**…$(ACT)_4$…	1 Compound perfect,	121	M	1		AF359971
	R: AATGATAACGGTAATAATGG	$(ATT)_3(ATCATT)_3AG(TAT)_3$…	2 perfect, 1 imperfect					

Continued

Table 2. Continued

Microsatellite clones[a]	Forward & Reverse Primers (5′→3′)	Repeat motifs[b]	Category (Weber, 1990)	Expected size (bp)	P[e]	# of alleles	%H_o^h	GenBank Accession #
TUMXLv10.224	F: CATCAGCTGTATTCCTACCC R: TAGCCAAAAGTAAACATGCC	…$(TA)_3$…**$(GTT)_3$**…	2 Perfect	153	N			AF359972
TUMXLv10.228	F: CCCATCTCTTTCTTTCTCGC R: GGAAGCGTTGAAAGATGAAC	…$(CT)_3$…**$(CT)_3$**…**$(TC)_3GN(TC)_3$**…**$(CT)_4$**…$(CA)_3$…	2 Perfect, 1 imperfect, 2 perfect	255	N			AF359973
TUMXLv10.234	F: TGGTCTGAACAAGAGGAAAG R: GATTTAGCTGCATACCATGTG	**…$(CA)_3$**…**$(CA)_4T(AC)_3(ACGCAC)_7$**…**$(AT)_3$**…$(TA)_3$…$(AT)_3$…$(AAT)_3$…	1 Perfect, 1 compound imperfect, 4 perfect	180	N			AF359974
TUMXLv10.237	F: ATTTCCCTAGATTTTGCCAG R: GTGATTAGGGCGATAATGG	…**$(TA)_3$**…**$(CAT)_3$**…$(TA)_3$…$(TTA)_4$…$(ATT)_3$…	5 Perfect	152	N			AF359975
TUMXLv10.238[c]	F: GATGCAACTGCITTCCTG R: GGCCAAGATAATITTATTTCCC	…$(GT)_3$…**$(GT)_3$**…**$(CTTT)_3$**…$(AT)_4GT(AT)_3$…$(TG)_3TA(TG)_5$…$(GT)_7$	3 Perfect, 2 imperfect, 1 perfect	207	P	4	75	AF359976
TUMXLv10.255	F: CTAAATAAATCACGGGTTGGG R: CCTTCTGGTTTACTGTTGAGGC	…**$(AT)_3$**…	Perfect	213	P	2	100	AF359977
TUMXLv10.264	F: GGACTATAATAACAATAATAGCCGT R: TTATGAGAGCAATTCGGACAA	…**$(ATT)_3$**…**$(TTA)_4$**…	2 Perfect	193	N			AF359978
TUMXLv10.278[c]	F: CAAGATGGAAGTGGATAGTG R: AAGATTCGTACTATITGCCG	…**$(ATT)_3$**…$(CT)_3TC(CT)_6T(TC)_3$…$(CT)_3CA(CT)_3$…$(TC)_{17}$…$(TC)_3$…$(TC)_3$	1 Perfect, 2 imperfect, 3 perfect	210	P	5	67	AF359980
TUMXLv10.283	F: AAAATATGCCGATGACAGGC R TAGTTACACTCGTCGCCCAC	…**$(TG)_3$**…	Perfect	143	M	1		AF359982
TUMXLv10.284	F: TCTTTAAAGGTCAGGTAAAGG R CGGCCAGACTCCACAACTAC	…**$(AC)_3$**…**$(AT)_3$**…	2 Perfect	205	P	3	67	AF359983
TUMXLv10.288[d]		…$(TAACC)_7$…	Perfect					AF359984
TUMXLv10.291	F: CCCTCAAACAGTCGCAGTG R GTTGGGTGAGTCTTTAGGCG	…**$(AT)_3$**…	Perfect	140	N			AF359985
TUMXLv10.295[g]	F: CATGTTTCCGGTTGTATATTCTG R GTTCAGTAGGTAGGAGT	…$(GA)_4$…**$(TCT)_5$**$CT(CTGT)_{15}(CT)_{24}(CA)_{23}$…	1 Perfect, 1 compound imperfect	192	N			AF359986
TUMXLv10.304	F: TCTTCCCTCCCCTTGTAACC R: CGCTGTCTCATTCTTCACCC	…**$(GC)_3$**…**$(CT)_3$**…$(GC)_4$…	3 Perfect	237	M	1		AF359987
TUMXLv10.311	F: CATCCACTTCTTCTCGTACCATC R: TCTCCATCCAGGTTCTGGG	…$(TA)_3$…**$(AG)_3$**…	2 Perfect	105	M	1		AF359988
TUMXLv10.312	F: ATACGAAACACCCCATCCC R: GTGGTCTTACCTCGTGGCTC	…$(AG)_{30}$…**$(AG)_5$**…	2 Perfect	179	P	2	44	AF359989
TUMXLv10.318[c]	F: CATCCTTATTATTGATACTGTTGC R AATGTCGAGATAGGAAGAG	…**$(TC)_5$**…[f]**$(CT)_4$**…$(TC)_{31}$	3 Perfect	93	P	3	38	AF359990

TUMXLv10.323[c]	F: CACCATTACTCTTATCCTTAC R GGAGGTGATTTTAAGATGTGC	…(TTA)$_3$…(ATT)$_4$…(TTA)$_3$…**(ATT)$_4$**…**(TTA)$_3$(TCA)$_3$TTA** **TT(ATCATT)$_3$**…(AAAC)$_3$…(AAAT)$_3$…(AC)$_5$… (TAT)$_4$…(CT)$_8$…(CT)$_{15}$	4 Perfect, 1 compound imperfect, 6 perfect	225	P	3	92	AF359993
TUMXLv10.324[c]	F: ATTCCGTGTTCTATGGTCTG R: CGCAGTGAAATAAAAGGAAG	…(AT)$_5$…**(GAA)$_3$**…(TTA)$_{22}$	3 Perfect	155	P	3	33	AF359991
TUMXLv10.340	F: GCATTGACTAGGCCTATATC R: GCCATGTTTACATCACCCAG	…**(AT)$_3$**…**(GT)$_4$**…(GT)$_3$…	3 Perfect	243	M	1		AF359994
TUMXLv10.341	F: CATATGTATCTGCCCTCGAC R: TAGGATGGTGGGTAGAGTTG	…**(TCCC)$_3$**…**(CCT)$_3$**…**(CT)$_3$**…(CCCT)$_3$…	4 Perfect	185	P	2	0	AF359995
TUMXLv10.343	F: CTTCCACATTCCCTATCTTC R: CATTCTTACATTCGACTGAGC	…**(TTC)$_4$**…**(TTC)$_4$**…(GT)$_4$…	3 Perfect	251	P	4	67	AF359996
TUMXLv10.349[c]		…(AT)$_4$AC(AT)$_4$…(TAAA)$_3$(TA)$_{10}$CA(TA)$_2$AG(TA)$_5$ (CA)$_9$T(TA)$_4$T(TA)$_{24}$(CA)$_{25}$(TA)$_{21}$G(TA)$_4$	1 Imperfect, 1 compound imperfect					AF359997
TUMXLv10.359	F: AATGAAGGTAACAGCCTCGC R: ACCCTGTTTGTAAAAATAGATATCCG	…**(AC)$_{40}$AT(AC)$_3$**…**(TA)$_3$**…(TA)$_3$…	1 Imperfect, 2 perfect	219	P	5	67	AF359998
TUMXLv10.363	F: TGAAGACCTGATAACTGATACGC R: TGTAGGAGTAGATGGTTTTCGTG	…**(CT)$_5$CC(CT)$_{14}$CCCTCT(CT)$_9$CC(CT)$_{11}$**…	Imperfect	284	P	3	38	AF359999
TUMXLv10.364	F: TGAAAGCATTCTGGTAAGGC R: GAATAAAACAAGGGGTGAGGG	…**(AT)$_3$**…**(AT)$_4$**…	2 Perfect	299	M	1		AF360000
TUMXLv10.368[c]		…(AC)$_4$AA(AC)$_2$AA(AC)$_M$G(CA)$_6$(TACACA)$_5$(CA)$_{32}$ TA(CA)$_{14}$…(AC)$_{30}$(AT)$_{26}$(AC)$_4$(CA)$_3$	1 Imperfect, 1 compound perfect					AF360001
TUMXLv10.384[c]		…(GT)$_3$CTTGTGC(AT)$_5$(ATAC)$_3$	Compound imperfect					AF360002
TUMXLv10.411	F: AGCACCTAGCACTTGCTGAAC R: AGAGACTCACATCCTCATCCTC	…**(TC)$_3$**…**(AAT)$_3$**…**(TAA)$_{12}$C(AAT)$_4$**…	2 Perfect, 1 imperfect	184	P	7	83	AF360004
TUMXLv10.447	F: ATACAGGCAGGCAGACAG R: GGTGTGAAGTGTGCAAATG	…**(CAGA)$_4$**…**(CA)$_3$**…	2 Perfect	219	N			AF360006
TUMXLv10.455[c]	F: AGAGTAGAAGAGGCAGGGCG R: GTCAAGAAGCAGGAAGGGTG	…**(CT)$_7$**…**(CT)$_{10}$**…**(TC)$_5$CC(TC)C(CT)$_3$**… **(CT)$_4$TT(CT)CG(CT)CC(CT)$_5$**…**(CT)$_3$**…	2 Perfect, 2 imperfect, 1 perfect	284	P	4	75	AF360007
TUMXLv10.481	F: CATAAGACTGCACACGTAGCG R: TTTAAAACGTGGTGTTCTGTGG	…(AT)$_4$…**(AT)$_3$**…	2 Perfect	125	M	1		AF360009
TUMXLv10.484	F: ACATCTGGTTGGTCTGAGGC R: GGAGTCGGTATC	…**(TC)$_3$**…	Perfect	237	P	4	33	AF360010

[a]Nomenclature for microsatellites is as follows: TU is Tufts University, followed by the initials of the researcher that cloned or characterized the microsatellite, (e.g., MX is Meehan Xu), the species name (Lv is *Penaeus (Litopenaeus) vannamei*) and clone number.

[b]Different microsatellites within a clone are separated by (…). Motifs in boldface indicate repeats flanked by the primers selected for analysis.

[c]There were not enough flanking sequences to design primers for all the motifs included in the sequence. However, primers may have been designed from a single or combined motifs within the sequence.

[d]Some TAACC motifs found in other clones have been genotyped and many of them were monomorphic (Alcivar-Warren et al., 2002).

[e]P, polymorphic; M, monomorphic; N, need further optimization (too many bands, no amplification, etc).

[f]Part of this motif was included in the design of the primer.

[g]There were too many Ns in middle of sequence—only partial sequence was submitted to GenBank.

[h]Observed Heterozygosity = Total Number of Heterozygotes divided by the Total Number of Samples (n = 3 to 20). All samples that amplified 1 band were considered homozygotes.

$(AT)_{15}$, $(GC)_{15}$, $(CT)_{15}$, $(TAT)_{10}$, $(CTC)_{10}$, $(CTTT)_8$, and $(TGTA)_8$] using γ-^{32}P ATP and the 5′-end labeling exchange reaction (Gibco). Filters were washed once in solution I (0.2% SDS, 2× SSC) for 15 minutes at room temperature, once in solution II (0.1% SDS, 1× SSC) for 15 minutes at room temperature, and continued to wash in solution II once at 37°C and one to two times at 42°C for 20 minutes until very low background radioactive signals were detected. Membranes were dried, exposed to film for 2 to 3 hours, and aligned to the numbered LB plates to identify the number of positive clones. Filters were stripped of the previous probe by first placing the membranes in molecular biology grade water for 2 to 3 minutes, and then adding a boiling solution of 0.1× SSC and 0.1% SDS, and were shaken for 15 minutes. Positive clone sequences have been deposited in GenBank (accession numbers AF359944–AF360116).

Characterization of Microsatellites

Microsatellites were divided into 3 categories: perfect, imperfect, and compound (Weber, 1990). All motifs with 3 or more repeats were counted as microsatellites. To compare with microsatellite frequencies reported in other studies, motifs with 5 or more repeats and 10 or more repeats were also counted.

Microsatellite Amplification, Scoring, and Calculation of PIC Values

The Primers3 program (Rozen and Skaletsky, 2002), as well as visual editing, was used to design 136 oligonucleotide primer sets flanking one or more motifs within a clone. Primer sets chosen were based on the uniqueness of sequences and percentage of GC content. Primers were synthesized (Operon Technologies Inc.) and used to amplify alleles in DNA (100 ng) from 26 cultured SPF shrimp (Alcivar-Warren et al., 2002). Polymerase chain reaction (PCR) mixture (25 µl) containing 100 ng DNA, 7.5 ng of γ-^{32}P-ATP–labeled reverse primer, 50 ng of forward primer, 2.0 mM of $MgCl_2$, 0.2 mM of dNTPs, 2.5 U of *Taq* polymerase (Promega) and 1× buffer. The thermal cycler (PTC-100, MJ Research) profile was 94°C for 3 minutes, 94°C for 1 minutes, 52°C for 1 minute, and 72°C for 2 minute, and it ran for 21 cycles (Wolfus et al., 1997). Amplified products were electrophoresed in polyacrylamide gels and visualized by autoradiography. Samples were run next to a known sequence (Garcia et al., 1996) to determine size. A microsatellite was regarded as polymorphic when the frequency of the most common allele is equal to or less than 0.99 (Nei, 1987). Polymorphism information content (PIC) was calculated as in Botstein et al. (1980).

Results and Discussion

Microsatellite-Containing Clones in Genomic Libraries

A total of 1479 positive clones were obtained after probe hybridization of the genomic library. The distribution of positive clones is summarized in Table 1. Microsatellite-containing clones were first identified in treatment 3 (vector-target ratio, 1:0.6) with 1 positive clone and increased to 116 clones in treatment 10 (vector-target ratio, 1:10). The 1:10 ratio provided more recombinant clones (n = 509) than 1:1 ratio (n = 80), indicating the importance of optimization of ligation conditions, as suggested by Ausubel et al. (2002).

A total of 251 clones tested positive to the dinucleotide, trinucleotide, and tetranucleotide, probes (Table 1), of which 173 (68.9%) contained microsatellites, 48 (19.2%) could not be sequenced, 16 (6.4%) did not contain microsatellites, and 14 (5.6%) were identical to other clones. Results indicated that only 173 (11.7%) of the 1479 positive clones actually contained microsatellite motifs after probe hybridization and sequencing.

Distribution of Microsatellites

In the 173 clones there were 588 microsatellites that consisted of 433 dinucleotide, 139 trinucleotide, 40 tetranucleotide, 35 pentanucleotide, 10 hexanucleotide, and 1 nanonucleotide motifs, alone or in combination, with 3 or more repeats (Table 2; Figure 1, B). Most of the motifs (n = 658) consisted of 3 or more repeats, whereas 223 motifs had 5 or more repeats and 104 motifs consisted of 10 or more repeats (Figure 1, A). Accordingly, based on the 8 probes used, the most abundant di-, tri-, tetra-, penta-, and hexanucleotide motifs were $(CT)_n$, $(ATT)_n$, $(CTTT)_n$, $(TAACC)_n$, and $(ATTATC)_n$ (Figure 1, B).

Dinucleotides

Relative to other species, dinucleotide repeats in *P. vannamei* are short, as reported in *Drosophila melanogaster*

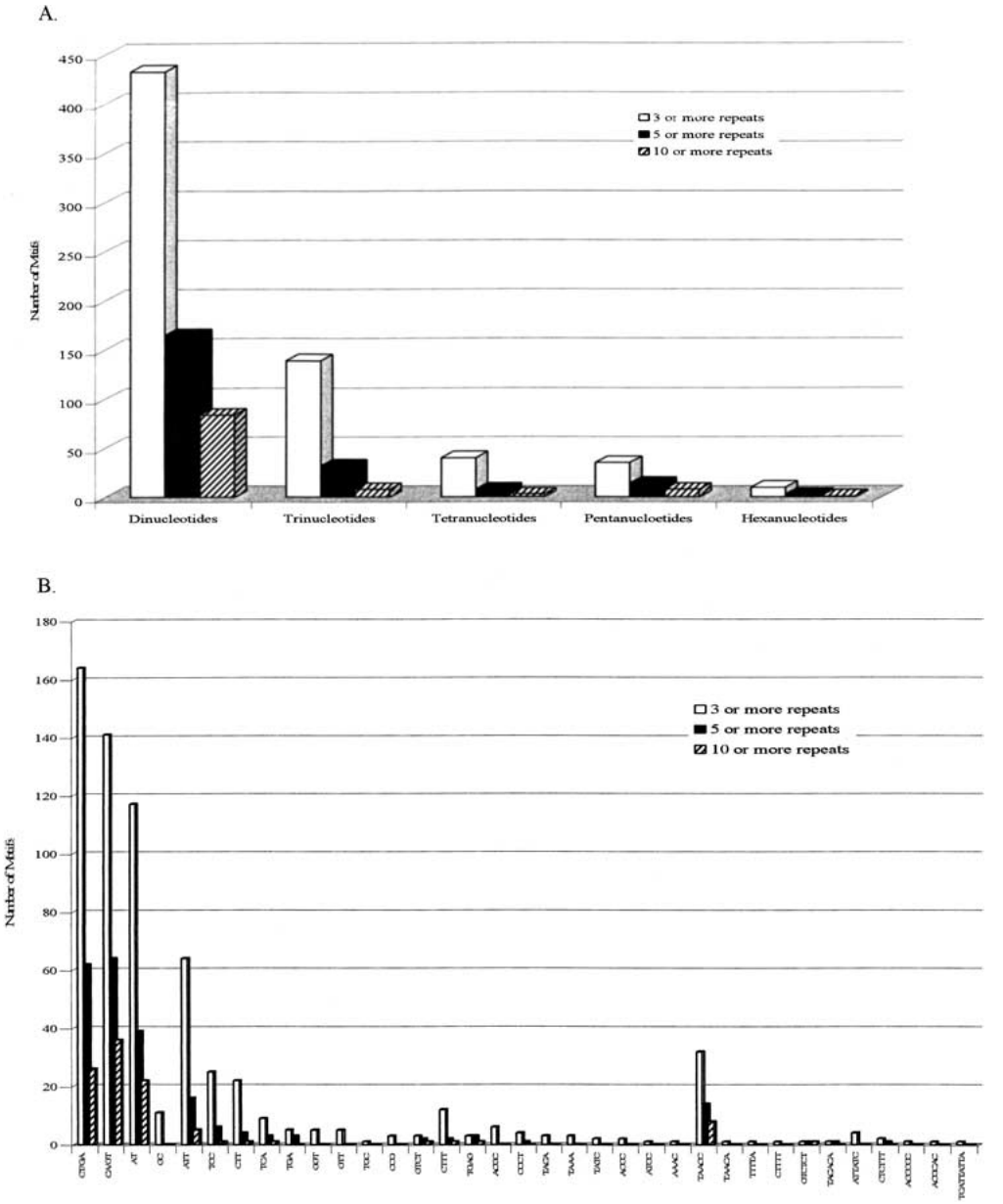

Figure 1. Summary of di-, tri-, tetra-, penta-, and hexanucleotide microsatellites (**A**) and core motifs (**B**) found in microsatellites with 3 or more, 5 or more, and 10 or more repeats, based on the methodology used in this study. Microsatellites were isolated from a genomic library of *Penaeus* (*Litopenaeus) vannamei* after probe hybridization as indicated in the "Materials and Methods" section.

(Schug et al., 1998). $(CT)_n$ was the most abundant ($n = 64$) microsatellite motif in *P. vannamei,* followed by $(GT)_n$ ($n = 141$), $(AT)_n$ ($n = 117$), and $(CG)_n$ ($n = 11$). These results are similar to those reported from another library of *P. vannamei* (Garcia and Alcivar Warren, 1996) and in hymenopteran species like the yellowjacket wasp, and humble bee (Thoren et al., 1995). In *D. melanogaster,* $(GT)_n$ was the most abundant microsatellites in arrays of 5 or more repeats, followed by $(TA)_n$ (Schug et al., 1998).

Trinucleotides

A considerable number of trinucleotide microsatellites were found in this study. TAT ($n = 64$) was the most abundant motif, followed by CTC ($n = 25$), CTT ($n = 22$), CAT ($n = 9$), and ACT ($n = 5$), among others. The abundance of the TAT and CTC repeats can partially be attributed to their use as probes. $(CTT)_n$ was also one of the first microsatellite motifs isolated from a randomly amplified polymorphic DNA (B20) marker in *P. vannamei* (Garcia et al., 1996).

Tetranucleotides

The most abundant tetranucleotide microsatellite found in *P. vannamei* is $(CTTT)_n$. This may be attributed to its use as a probe. However, this should not be taken as a general rule, because the other tetranucleotide (TGTA) used as a probe was not even the second most abundant tetranucleotide. $(CTTT)_n$ was also isolated in *B20* locus *(M1* microsatellites) and is a highly polymorphic marker (Garcia et al., 1996). This $(CTTT)_n$ microsatellite (*M1*) has also been successfully used to study genetic diversity of wild and cultured populations, track the pedigree of the USMSFP breeding program, and search for allele frequency differences in TSV-resistant and TSV-susceptible shrimp (Wolfus et al., 1997; Z. Xu et al., unpublished results).

Pentanucleotides

A large number of TAACC-containing clones were found in this study. These were not found in another *P. vannamei* genomic library obtained after probe hybridization (Garcia and Alcivar-Warren, 1996) or in sequences of *P. monodon* obtained after direct sequencing *(*Xu et al., 1999). However, similar pentanucleotide repeats were reported in *P. vannamei* by Bagshaw and Buckholt (1997), but our sequences show a more variable structure of the core motif. Variable core motifs of pentanucleotide $(TAACC/TTAGG)_n$ have also been found in tick *Boophilus annulatus* (AF50888), human (AC018606, AL358113), silkworm *Bombyx mori* (D13554), Mediterranean fluor moth *Anagasta kuehniella* (X70283), and fish *Pugu rubripes* (AF064564).

Categories of Microsatellite Motifs

Most shrimp microsatellites were categorized as perfect (79.6%), followed by imperfect (10.5%), compound imperfect (6.6%), and compound perfect (3.2%). These results are similar to those found for *P. vannamei* (Garcia and Alcivar-Warren, 1996) and *P. monodon* (Xu et al., 1999). However, Tassanakajon et al. (1998) found that imperfect dinucleotide microsatellites were the most abundant in *P. monodon.* Results from fish and mammalian species also indicated that perfect microsatellites were most abundant within the genome (Weber, 1990; Beckmann and Weber, 1992; Brooker et al., 1994; Crooijmans et al., 1997).

The number of uninterrupted repeats in *P. vannamei* microsatellites ranged from 3 to 57 (Table 2), with the majority consisting of short dinucleotide repeats.

Table 3. Distribution and Frequency of Microsatellite Motifs with Three or More Nucleotide Repeats in *Penaeus (Litopenaeus) vannamei*

	Dincleotides[a]		Trinucleotides		Tetranucleotides		Pentanucleotides[a]		Hexanucleotides[a]		Nanonucleotides[a]		Total	
Repeat of motifs	Number	Frequency (1/kb)	Number	Frequency (1/kb)	Number	Frequency (1/kb)	Number	Frequency (1/kb)	Number	Frequency (1/kb)	Number	Frequency (1/kb)	Number	Frequency (1/kb)
Three or more repeats[b]	433	1/1.43	139	1/4 .48	40	1/15.46	35	1/17.66	10	1/61.82	1	1/618.22	658	1/0.94
Five or more repeats	165	1/3.75	32	1/19.32	8	1/77.27	14	1/44.16	3	1/206.07	0	–	222	1/2 78
Ten or morer repeats	84	1/7.36	8	1/77.27	3	1/206.07	8	1/68.69	1	1/618.22	0	–	104	1/5.94

[a]These microsatellite repeats may have tested positive to one or more of the di-, tri-, and tetranucleotide probes.

[b]The estimated frequency of microsatellites was obtained by dividing the total length of the *Penaeus (Litopenaeus) vannamei* genomic library (618,222 base pairs = 1,479 × estimated average insert length 418 bp) by the total number of repeats then divided by 1000.

Table 4. Usability of 110 *Penaeus (Litopenaeus) vannamei* Microsatellites with Different Motif Lengths[a]

	Motifs with <6 repeats	Motifs with <10 repeats	Motifs with >10 repeats	Total
Polymorphic	51 (55%)	13 (14%)	29 (31%)	93
Monomorphic	15 (94%)	1 (6%)	0	16

[a]From a total of 136 primer sets tested, 27(N, Table 2) of which to be optimized

Frequency of Microsatellites

The frequencies of different microsatellite motifs in *P. vannamei* are shown in Table 3. Among the dinucleotides, frequency of $(GT)_n$, $(CT)_n$, $(AT)_n$, and $(CG)_n$ with 3 or more repeats was 1 in 4.38 kb, 1 in 3.77 kb, 1 in 5.28 kb, and 1 in 56.2 kb, respectively. The frequency of *P. vannamei* microsatellites reported here is relatively high when compared with that in *P. monodon* (Tassanakajon et al., 1998; Brooker et al., 2000). For instance, the frequency in *P. vannamei* of $(GT)_n$ and $(CT)_n$ microsatellite motifs with 3 or more repeats was 1 in 4.38 kb and 1 in 3.77 kb; 5 or more repeats was 1 in 9.66 kb and 1 in 9.97 kb; 10 or more repeats was 1 in 17.17 kb and 1 in 23.78 kb, respectively. In *P. monodon,* Tassanakajon et al. (1998) found a lower frequency of $(GT)_n$ (1 in 93 kb) and $(CT)_n$ (1 in 164 kb) among microsatellites with 6 or more repeats. Brooker et al. (2000) also reported a low frequency of $(GT)_n$ (1 in 164 kb) and $(CT)_n$ (1 in 1200 kb) in *P. monodon.* In other species, the frequencies of $(GT)_n$ and $(CT)_n$ were 1 in 23 kb and 1 in 76 kb for brown trout (Estoup et al., 1993a), 1 in 139 and 1 in 87 in flat oyster (Naciri et al., 1995), 1 in 15 kb for GT in honeybee (Estoup et al., 1993b), and 1 in 8 kb and 1 in 2.5 kb for yellowjacket wasp (Thoren et al., 1995). McConnell et al. (1995) and Slettan et al. (1993) reported average frequencies of $(GT)_n$ repeats every 24 to 35 kb and every 90 kb, respectively, for Atlantic salmon. The frequencies of $(GT)_n$ and $(CT)_n$ in the present study are higher than in most studies and similar to the yellowjacket wasp and honeybee, even when we only consider motifs with 10 or more repeats. Furthermore, the density of microsatellites in *P. vannamei* genome may have also been underestimated due to probe hybridization.

Microsatellite Polymorphism

Out of the 173 microsatellite-containing clones, 128 (74.0%) had enough flanking sequences to design primers covering all the motifs included in the clones (Table 2). In an effort to increase the number of polymorphic markers for mapping studies, primer sets were designed to include single or multiple motifs with 3 or more repeats, which allowed us to design primer sets from 136 (78.6%) of the sequences. Ninety-three (68.0%) of the 136 primer sets successfully amplified scorable, polymorphic bands in cultured *P. vannamei,* with allele sizes ranging from 98 bp to 470 bp, and PIC values ranging from 0.195 to 0.871. Among the 93 polymorphic microsatellites, 51 (55.3%) contained single or multiple motifs of less than 6 repeats each (Table 4, Figure 2). The remaining primer sets either amplified many unscorable bands or did not amplify at the annealing temperature we used and need to be further optimized.

Our results showed that a high percentage (78.6%) of clones contained enough flanking sequences to design primers for genotyping. Xu et al. (1999) and Vonau et al. (1999) also reported that 87% and 70% of their microsatellite-containing clones had long enough flanking sequences to design primers in *P. monodon* and *P. stylirostris,* respectively. These findings were not consistent with the results from other studies in *P. monodon* (Tassanakajon et al., 1998; Brooker et al., 2000) and *P. japonicus* (Moore et al., 1999). Further characterization of annealing temperature for the 27 (N) clones listed in Table 2 may increase the number of useful markers.

Most studies have used 6 repeats (Stallings et al., 1991; Thoren et al., 1995; Tassanakajon et al., 1998) or 10 repeats (Tautz, 1989; Beckmann and Weber, 1992) to identify microsatellites. However, Schug et al. (1998) used 5 repeats as criteria to search for *Drosophila* microsatellites in GenBank. McConnell et al. (1995) and Slettan et al. (1993) included microsatellites with 4 to 6 repeats in Atlantic salmon *(Salmo salar)*, Xu et al. (1999) identified all microsatellites with 3 or more repeats in *P. monodon,* and Naciri et al. (1995) included a tetranucleotide with 3 repeats in flat oyster. It is known that informativeness of microsatellite markers increases with the number of repeats (Weber, 1990). However, there is no reason to exclude microsatellites with less than 6 repeats if they do show polymorphisms. Strassman et al. (1997) analyzed the relationship between repeat length and heterozygosity and

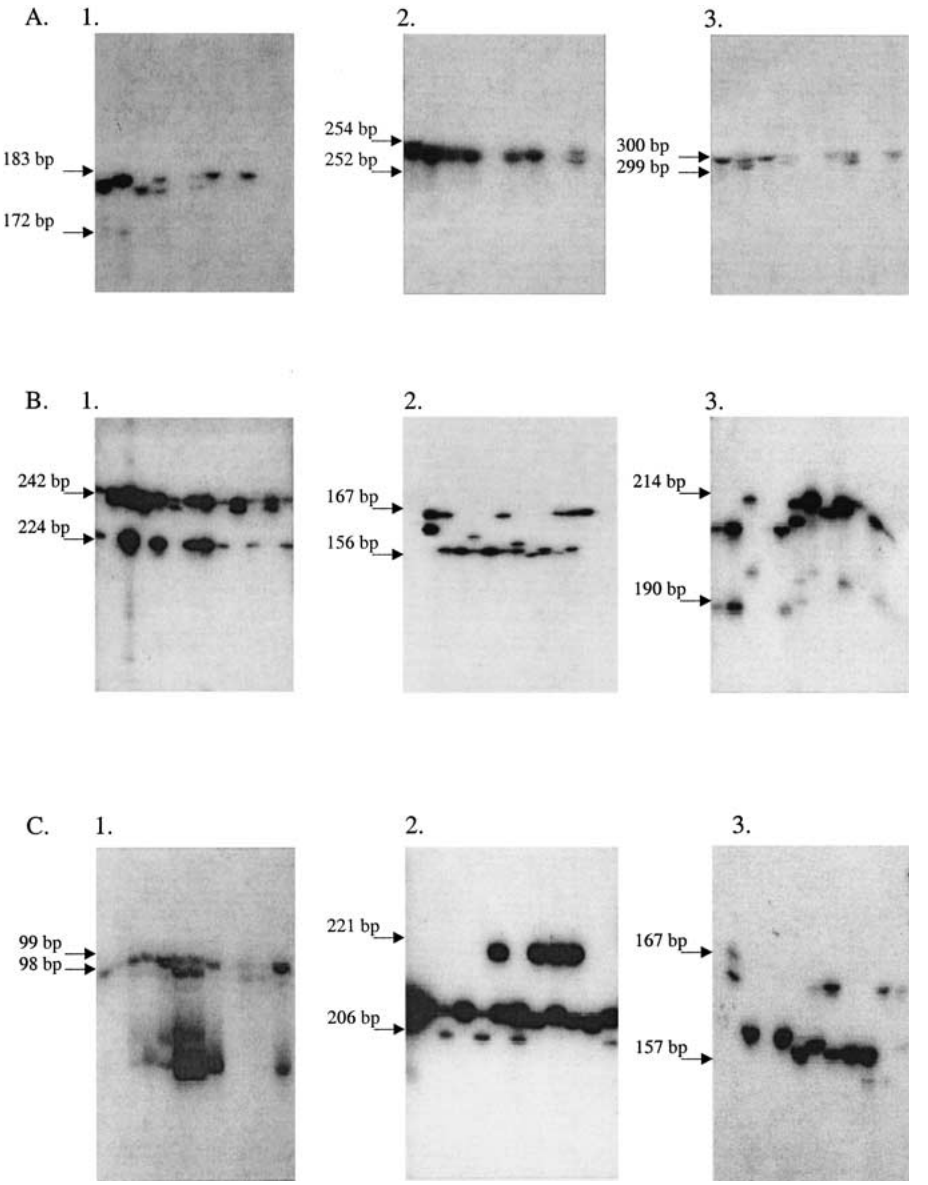

Figure 2. Scanned autoradiograms of polymorphic microsatellite motifs in cultured, SPF Penaeus (Litopenaeus) vannamei. The approximate range of product size (in base pairs) for each autoradiogram is indicated with arrows. **A:** Microsatellites containing only dinucleotide motifs with less than 6 repeats. (1) TUMXPv10.312 $[AG]_5$; (2) TUMXPv9.149 $[(TA)_3\ldots(TA)_3]$, and (3) TUMXPv8.224 $[(AT)_4\ \ldots(AC)_3\ldots(TG)_3]$. **B:** Microsatellites containing single or multiple trinucleotide motif with less than 6 repeats. (1) TUMXPv10.176 $[(GT)_4\ldots(TTC)_3]$; (2) TUMXPV8.256 $[(AAT)_4]$; and (3) TUMXPv9.77 $[(ATA)_3]$. **C:** Microsatellites containing single or multiple motifs with tetranucleotides and pentanucleotide motifs with less than 6 repeats. (1) TUMXLv5.35 $[(CTTT)_3]$; (2) TUMXPV10.238 $[(GT)_3\ldots(CCCT)_3]$; and (3) C. TUMXPv9.28 $[(ATC)_3\ldots(CT)_3\ldots(CTTT)_3TTT(CTTT)_3]$.

concluded that it is worth pursuing microsatellites with as few as 5 perfect repeats. Orti et al. (1997) also found that repeat number of a highly polymorphic $(CA)_n$ microsatellite locus varied from 5 to 11. Xu et al. (1999) amplified 11 polymorphic microsatellites for *P. monodon* and 3 of them had less than 6 repeats. In the present work, many of the short motifs with less than 6 repeats were polymorphic. Out of the 93 polymorphic markers, 51 (55%) contained 1 or more repeat motifs with less than 6 repeats (Table 4). Our results indicate that designing primers to flank one or more motifs of less than 6 repeats, which may be ignored by conventional methods, can greatly increase the number of useful markers. The observed heterozygosity ranging from 10% to 100% shows that these could be useful markers. Samples with a single allele were regarded as homozygous although they could be one amplified allele and one null

allele (Pemberton et al., 1995). Further analysis with a large family or newly designed primers will be needed to identify null alleles. Eight (53.3%) out of 15 single-motif microsatellites containing less than 6 dinucleotide repeats were polymorphic, suggesting that the PIC value is not always 0 for microsatellite with less than 10 repeats, as reported by Weber (1990). If we had only chosen microsatellite with motifs of 10 or more repeats, the number of polymorphic markers would have decreased from 93 to 29 (Table 4).

In summary, a considerable number of *P. vannamei* clones that contained microsatellites were obtained by optimizing the vector–shrimp DNA ligation conditions and using probe hybridization. Many of these clones had large enough flanking sequences to design primers. Designing primers that flank one or more separated motifs, even with less than 6 repeats, greatly increased the number of polymorphic markers obtained. All polymorphic markers identified here are being used along with other markers to construct a linkage map for penaeid shrimp.

Acknowledgments

Thanks to Steve Arce, Brad Argue, Fernanda Calderon, William Carr, Shaun Moss, Monica Traub, and Jim Sweeney, from the Oceanic Institute, Honolulu, Hawaii, for access to shrimp tissue samples, Kelly Johnson for assistance with plasmid DNA isolation, and Kireina Bell for editing the cloned sequences. This work was supported by the U.S. Department of Agriculture, through a grant (#95-388-1424) to the USMSFP. Partial support was received from the Department of Environmental and Population Health at Tufts University School of Veterinary Medicine.

References

Alcivar-Warren, A., Xu, Z., Meehan, D., Fan, Y., and Song, L. (2002). Shrimp genomics: development of a genetic map to identify QTLs responsible for economically important traits in *Litopenaeus vannamei.* In: *Aquatic Genomics: Steps Toward a Great Future,* Shimizu, N., Aoki, T., Hirono, I., and Takashima, F., eds. Tokyo, Japan: Springer-Verlag, 61–72.

Argue, B.J., and Alcivar-Warren, A. (1999). Genetics and breeding applied to the penaeid shrimp farming industry. In: Bullis, R.A., and Pruder, G.D., eds. *Controlled and Biosecure Production Systems:* Preliminary Proceedings of a Special Session on Evolution and Integration of Shrimp and Chicken Models, Honolulu, Hawaii: Oceanic Institute, 29–54.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (2002). *Current Protocols in Molecular Biology*. Waltham, Mass.: John Wiley and Sons, Inc.

Bagshaw, J., and Bucholt, M. (1997). A novel satellite/microsatellites combination in the genome of the marine shrimp, *Penaeus vannamei. Gene* 184:211–214.

Baldwin, J.D., Bass, A.L., Bowen, B.W., and Clark, W.H. (1998). Molecular phylogeny and biogeography of the marine shrimp *Penaeus. Mol Phylogenet Evol* 10:399–407.

Ball, A.O., Leonards, S., and Chapman, R.W. (1998). Characterization of $(GT)_n$ microsatellites from native white shrimp (*Penaeus setiferus*). *Mol Ecol* 7:1251–1253.

Beckmann, J.S., and Weber, J.P. (1992). Survey of human and rat microsatellites. *Genetics* 12:627–631.

Botstein, D., White, R.P., Skolnick, M., and Davis, R.W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Anim Genet* 32:314–331.

Brooker, A.L., Cook, D., Bentzen, P., Wright, J.M., and Doyle, R.W. (1994). Organization of microsatellites differs between mammals and cold-water teleost fishes. *Can J Fish Aquat Sci* 51:1959–1966.

Brooker, A.L., Benzie, J.A.H., and Blair, D. (2000). Population structure of the giant tiger prawn *Penaeus monodon* in Australian waters determined using microsatellites markers. *Mar Biol* 136:149–157.

Carr, W.H., Fjalestad, K.T., Godin, D., Swingle, J., Sweeney, J.N., and Gjedrem, T. (1997). Genetic variation in weight and survival in a population of specific pathogen-free shrimp, *Penaeus vannamei.* In: *Diseases in Asian Aquaculture III*, Flegel, T.W., and MacRae, I.H., eds. Manila, Philippines: Fish Health Section, Asian Fisheries Society, 265–271.

Crooijmans, R.P.M.A., Bierbooms, V.A.F., Komen, J.,Van de Poel, J.J., and Groenen, M.A.M. (1997). Microsatellite markers in common carp (*Cyprinus carpio* P.). *Anim Genet* 28:129–134.

Estoup, A., Presa, P., Krieg, F., Vaiman, D., and Guyomard, R. (1993a). $(CT)_n$ and $(GT)_n$ microsatellites: a new class of genetic markers for *Salmo trutta* L. (brown trout). *Heredity* 71:488–496.

Estoup, A., Solignac, M., Harry, M., and Cornut, J.M. (1993b). Characterization of GT and CT microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris. Nucleic Acids Res* 21:1427–1431.

Garcia, D.K., and Alcivar-Warren, A. (1996). Identification and organization of microsatellites in *Penaeus vannamei* shrimp. In: *Proceedings of the XXVth International Conference on Animal Genetics,* Tours, France, July, 21–25, 1996, Abstract C061, 113.

Garcia, D.K., Faggart, M.A., Rhoades, L., Wyban, J.A., Carr, W., Ebert, K.M., Sweeney, J.N., and Alcivar-Warren, A. (1994). Genetic diversity of cultured *Penaeus vannamei* shrimp using three molecular genetic techniques. *Mol Mar Biol Biotechnol* 3:270–280.

Garcia, D.K., Dhar, A.K., and Alcivar-Warren, A. (1996). Molecular analysis of a RAPD marker (B20) reveals two microsatellites and differential mRNA expression. *Mol Mar Biol Biotechnol* 5:71–83.

Hearne, C.M., Ghosh, S., and Todd, J.A. (1992). Microsatellites for linkage analysis of genetic traits. *Trends Genet* 8:288–294.

Lightner, D.V., Redman, R.M., Poulus, B.T., Nuna, L.M., Mari, J.L., and Hasson, K.W. (1997). Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. *Rev Sci Tech Off Int Epiz* 16:146–160.

Lotz, J.M., Browdy, C.L., Carr, W.H., Frelier, P.P., and Lightner, D.V. (1995). USMSFP suggested procedures and guidelines for assuring the specific pathogen status of shrimp broodstock and seed. In: *Swimming Through Troubled Waters* (Proceedings of the Special Session on Shrimp Farming), Browdy, C.L., and Hopkins, J.S., eds. Baton Rouge, La.: World Aquaculture Society, 66–75.

McConnell, S.K., O'Reilly, P., Hamilton, L., Wright, J.M., and Bentzen, P. (1995). Polymorphic microsatellites loci from Atlantic salmon *(Salmo salar)*: genetic differentiation of North American and European populations. *Can J Fish Aquat Sci* 52:1863–1872.

Moore, S.S., Whan, V.A., Davis, G.P., Byrne, K., Hetzel, D.J.S., and Preston, N. (1999). The development and application of genetic markers for the kuruma prawn *Penaeus japonicus. Aquaculture* 173:19–32.

Naciri, Y., Vigouroux, Y., Dallas, J., Desmarais, E., Delsert, C., and Bonhomme, F. (1995). Identification and inheritance of $(GA/TC)_n$ and $(AC/GT)_n$ repeats in European flat oyster *Ostrea edulis* (L.). *Mol Mar Biol Biotechnol* 4:83–89

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., and Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature* 405:1017–1024.

Nei, M. (1987). *Molecular Evolutionary Genetics.* New York, N.Y.: Columbia University Press, 176–207.

Orti, G., Pearse, D.E., and Avise, J.C. (1997). Phylogenetic assessment of length variation at a microsatellite locus. *Proc Natl Acad Sci USA* 94:10745–10749.

Ozaki, A., Sakamoto, T., Khoo, S., Nakamura, K., Coimbra, M.R., Akutsu, T., and Okamoto, N. (2000). Quantitative trait loci (QTLs) associated with resistance/susceptibility to infectious pancreatic necrosis virus (IPNV) in rainbow trout (*Oncorhynchus mykiss*). *Mol Genet Genom* 265:23–31.

Pemberton, J.M., Slate, J., Bancroft, D.R., and Barrett, J.A. (1995). Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Mol Ecol* 4:249–252.

Rozen, S., and Skaletsky, H.J. (2000). Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, Krawtz, S., and Misener, S., eds. Totowa, N.I.: Humana Press, 365–386. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Schug, M.D., Wetterstrand, K.A., Gaudette, M.S., Lim, R.H., Hutter, C.M., and Aquadro, C.F. (1998). The distribution and frequency of microsatellites loci in *Drosophila melanogaster. Mol Ecol* 7:57–70.

Slettan, A., Olsaker, I., and Lie, O. (1993). Isolation and characterization of variable $(GT)_n$ repetitive sequences from Atlantic salmon, *Salmo salar* L. *Anim Genet* 24:195–197.

Stallings, R.L., Ford, A.F., Nelson, D., Torney, D.C., Hildebrand, C.E., and Moyzis, R.K. (1991). Evolution and distribution of $(GT)_n$ repetitive sequences in mammalian genomes. *Genomics* 10:807–815.

Strassman, J.E., Barefield, K., Solis, C.R., Hughes, C.R., and Queller, D.C. (1997). Trinucleotide microsatellites loci for a social wasp, Polistes. *Mol Ecol* 6:97–100.

Tassanakajon, A., Tiptawonnukul, A., Supungul, P., Rhimphanitchayakit, V., Cook, D., Jarayabhand, P., Klinbunga, S., and Boonsaeng, V. (1998). Isolation and characterization of microsatellites markers in the black tiger prawn *Penaeus monodon. Mol Mar Biol Biotechnol* 7:55–61.

Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res* 17:6468–6471

Thoren, P.A., Paxton, R.J., and Estoup, A. (1995). Unusually high frequency of $(CT)_n$ and $(GT)_n$ microsatellites loci in a yellowjacket wasp, *Vespula rufa* (P.) (Hymenoptera: Vespidae). *Insect Mol Biol* 4:141–148.

Vonau, V., Ohresser, M., Bierne, N., Delsert, C., Beuzart, I., Bedier, E., and Bonhomme, F. (1999). Three polymorphic microsatellites in the shrimp *Penaeus stylirostris. Anim Genet* 30:234–235.

Weber, J.L. (1990). Informativeness of human $(dC\text{-}dA)_n$ $(dG\text{-}dT)_n$ polymorphisms. *Genomics* 7:524–530.

Wolfus, G.M., Garcia, D.K., and Alcivar-Warren, A. (1997). Application of the microsatellites technique for analyzing genetic diversity in shrimp breeding programs. *Aquaculture* 152:35–47.

Wright, J.M., and Bentzen, P. (1994). Microsatellites: genetic markers for the future. *Rev Fish Biol Fish* 4:384–388.

Xu, Z., Dhar, A.K., Wyrzykoswki, J., and Alcivar-Warren, A. (1999). Identification of abundant and informative microsatellites from shrimp *(Penaeus monodon)* genome. *Anim Genet* 30:1–17.