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CHARACTERIZATION OF 35 NEW MICROSATELLITE GENETIC MARKERS FOR THE PACIFIC WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI*: THEIR USEFULNESS FOR STUDYING GENETIC DIVERSITY OF WILD AND CULTURED STOCKS, TRACING PEDIGREE IN BREEDING PROGRAMS, AND LINKAGE MAPPING

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ABSTRACT A large number of polymorphic genetic markers are needed to examine genetic variation in wild and cultured penaeid species, trace pedigrees, and apply marker-assisted selection in breeding programs. The objectives of this study are to (1) isolate and characterize microsatellite genetic markers for the Pacific whiteleg shrimp, *Litopenaeus vannamei*, (2) demonstrate the usefulness of three randomly selected markers to examine allelic variation in wild and cultured shrimp populations, and trace the pedigree of two families from the breeding program of the US Marine Shrimp Farming Program (USMSFP); and (3) determine the potential usefulness of these microsatellites for linkage mapping. A total of 128 recombinant clones obtained from *Sau3* A-digested genomic libraries prepared from ovary of specific pathogen-free *L. vannamei* were sequenced; 86 of which contained simple sequence repeats (SSRs), or microsatellites, with three or more repeat motifs. The frequency of microsatellites with five or more repeats was estimated at 1/2.74 kb. The most abundant di-, tri, tetra-, penta-, and hexa-nucleotide motifs were $(CT)_n$, $(CCT)_n$ and $(CTT)_n$, $(CATA)_n$, $(CTTCT)_n$, and $(GAGATA)_n$. The octa-nucleotide $(CCCTCTCT)_3$ was also identified. Sixty-two primer sets flanking microsatellites with single or multiple motifs were designed and tested for polymorphism with a small test panel representing individuals of the mapping families being used to develop a linkage map for *L. vannamei* (*ShrimpMap*), and 35 of these (56.4%) were polymorphic. Three of these markers (TUGAPv1-3.224, TUGAPv5-7.33, and TUGAPv7-9.17) were used for estimating allele diversity of wild populations of Ecuador and Mexico and tracing the pedigree in two families of the USMSFP breeding program. A large number of alleles (21–31) and allele size range (95–275 bp) was observed in wild shrimp. There was a large allele size range difference at all three loci examined, being smaller in cultured shrimp (32–74 bp) than in wild shrimp (77–180 bp), suggesting null alleles or mutations. The presence of stuttering bands with marker TUGAPv5-7.33 made it difficult to score the wild shrimp from Mexico and suggest the need to first test for inheritance pattern of shrimp microsatellites before using them in population genetics, relatedness/kinship, and traceability studies. Allele segregation in cultured shrimp confirmed codominant inheritance of markers. Observed heterozygosity was 100% for all loci scored. Fourteen randomly selected polymorphic markers were further genotyped with the entire IRMF panel and 8 of these amplified with most of the individuals tested. Linkage analysis using CRIMAP with LOD score of 5.0 placed four of the markers (TUGAPv1-3.132, TUGAPv3-5.213, TUGAPv7-9.179, and TUGAPv7-9.95) in linkage groups LG6, LG5, LG13, and LG14, respectively, and four markers (TUGAPv3-5.271, TUGAPv3-5.391, TUGAPv7-9.94, and TUGAPv7-9.226) remained unlinked. In summary, 35 new microsatellites were developed for *L. vannamei*, some of which are useful for studies on genetic diversity of wild and cultured stocks, pedigree tracing in breeding programs, and linkage mapping. Moreover, some of the genomic sequences reported here had significant homology to hypothetical proteins of various organisms, known (e.g., reverse transcriptase) or unknown genes, or no homology to any sequence in the GeneBank database, suggesting that sequences from a genomic library can also provide valuable information in identifying functional markers in shrimp.

KEY WORDS: simple sequence repeats (SSRs), expressed sequence tags (EST), EST-SSRs, microsatellites, *Litopenaeus vannamei, ShrimpMap,* transposable elements, non-LTR retrotransposons, reverse transcriptase

INTRODUCTION

Various selective breeding programs for penaeid shrimp have been established to genetically improve cultured stocks (reviewed in Argue & Alcivar-Warren 1999), which may help eliminate overfishing in the wild (Naylor et al. 2000). One such program, developed by the U.S. Marine Shrimp Farming Program Consortium (USMSFP), maintains specific pathogen-free (SPF) captive populations of Pacific whiteleg shrimp, *Litopenaeus vannamei*, for distribution to shrimp producers (Lotz et al. 1995, Moss et al. 1999, Argue et al. 2002). The USMSFP first began domestication of *L. vannamei* free of Infectious Hypodermal and Hematopoeitic Necrosis Virus (IHHNV) (Lotz et al. 1995, Alcivar-Warren et al. 1997, Carr et al. 1997). Later, when Taura Syndrome Virus (TSV) emerged as a major problem for the industry (Lightner et al. 1997), the same stocks were used to selectively breed for TSV resistance and other economically important traits like high growth and survival under near zero water exchange and low salinity conditions (Argue & Alcivar-Warren 1999, Moss et al. 1999, Argue et al. 2002, Xu et al. 2003a). To better understand these traits and increase the rate of genetic improvement in shrimp breeding programs, the loci responsible for them need to be identified for further use in marker-assisted selection. To do this, a large number of highly polymorphic genetic markers are needed to develop a framework linkage map for shrimp.

Simple sequence repeats, or microsatellites, are the markers of choice for genetic analysis and gene mapping of agricultural species because of their abundance, high levels of polymorphism,

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Mendelian inheritance and codominant expression (Wright & Bentzen 1994, O'Reilly & Wright 1995, Ozaki et al. 2000). In shrimp, a small number of microsatellites has been developed for various penaeid species (Garcia et al. 1996, Bagshaw & Bucholt 1997, Ball et al. 1998, Tassanakajon et al. 1998, Vonau et al. 1999, Moore et al. 1999, Pongsomboon et al. 2000, Xu et al. 1999, Cruz et al. 2002, Maggioni et al. 2003, Meehan et al. 2003, Wuthisuthimethavee et al. 2003a) and have mostly been used in population genetic studies (Garcia et al. 1994, Brooker et al. 2000, Xu et al. 2001, Ball & Chapman 2003, Maggioni et al. 2003), genetic relationships (Xu et al. 2003a), pedigree tracking and genetic diversity in breeding programs (Wolfus et al. 1997, Moore et al. 1999, Vonau et al. 1999), and gene mapping efforts (Moore et al. 1999, Alcívar-Warren et al. 2002, Wuthisuthimethavee et al. 2003b). Additional markers are needed for differentiating stocks of breeding programs, tracking pedigrees, studying fitness and genetic diversity of natural populations, mapping quantitative trait loci in penaeid shrimp, and traceability of imported shrimp. The specific objectives of this study are (1) to isolate and characterize microsatellite genetic markers isolated from genomic libraries of SPF *L. vannamei*, (2) to demonstrate the usefulness of three randomly selected markers to examine allelic variation in wild and cultured shrimp populations, and trace the pedigree of two families from the selective breeding program developed by the USMSFP, and (3) to determine potential usefulness of these microsatellites for linkage mapping.

MATERIALS AND METHODS

Construction and Screening of Genomic Libraries

The procedures for library construction and screening were as performed by Mr. Doug Holder in the laboratory of Dr. Scott Davis, TX A&M University, College Station, TX (pers. comm.) with minor modifications. Briefly, 10 µg of ovary DNA was partially digested with 20 units of *Sau* 3A (Gibco BRL) restriction enzyme at 37°C for one hour. At the same time, the vector pBluescript II SK+ (Strategene) was digested with *Bam* HI (Gibco) following manufacturer's instructions. Digested DNA was electrophoresed on a 0.8% agarose gel in TAE (0.8 M Tris, 0.4 mM glacial acetic acid and 0.4 mM EDTA) (Garcia et al. 1994), and four different band ranges of approximately 100–300 (1–3) bp, 300–500 (3–5) bp, 500–700 (5–7) bp and 700–900 (7–9) bp were eluted using Spin-X columns (Costar, MA). DNA was precipitated with 3M sodium acetate (pH 5.2) and 100% ethanol. The 5′ phosphate groups were removed using 5 units of calf intestinal alkaline phosphatase (Promega) and 10 mM Tris-HCl at 37°C for 45 min. Proteins in the mixture (100 µl) were degraded by adding 2.5 µl of 0.1% SDS, 1 µl of 0.5 mM EDTA and 1 µl 10 mg/mL Proteinase K, and incubating at 55°C for 30 min, and removed with phenol/chloroform extraction. The four DNA fractions were each combined with an equal molar amount of digested pBluescript II SK+ vector and ethanol precipitated. The resulting DNA/vector pellet was ligated with T4 DNA ligase (Promega) at 15°C for 18 h and transformed into DH5α competent cells (Gibco) following manufacturer's instructions. Transformed cells were each grown on plates containing LB/ampicillin/IPTG/Bluo-Gal at 37°C overnight. The recombinant white colonies were streaked onto new plates, and also onto nylon membranes (MSI, Westboro, MA) and allowed to grow on the nylon filters for an additional 4–10 h. Filters were prehybridized in 20 mL of hybridization solution (5 × SSC, 0.5% SDS, 25 mM potassium phosphate [0.5 M KH_2PO_4, 0.5 M K_2HPO_4, pH 6.5] and 5 × Denhardts] and incubated for 1 h at 65°C. Probes were labeled using γ-^{32}P ATP and the 5′-end labeling exchange reaction (Gibco). Probes were sequentially labeled as follows: $(GT)_{15}$, $(CT)_{15}$, $(AT)_{15}$, $(CG)_{15}$, $(GTG)_5$ and a microsatellite, M2, found in previous work (Garcia et al. 1996). Probe hybridizations were performed at 37°C overnight using the same prehybridization solution. Filters were washed once in solution I (0.2% SDS, 2 × SSC) for 15 min at room temperature, once in solution II (0.1% SDS, 1 × SSC) for 15 min at room temperature and then once in solution II for 20 min at 42°C. The positively identified clones were grown overnight and DNA isolated following standard procedures (Garcia et al. 1996, Meehan et al. 2003). Filters were stripped of the previous probe by placing the membranes in a boiling solution containing 0.1% SDS, 0.1 × SSC and gently shaken for 30 min.

DNA Sequencing, Microsatellite Characterization and Homology Searches

Positive plasmid clones were sequenced using either a manual protocol (Promega protocol VI of the *fmol* sequencing kit) or an ABI 377 DNA sequencer at the DNA Sequencing Facility of Tufts University, Boston, MA. The sequencing reaction and cycle conditions for manual protocol were as suggested by the manufacturer: 95°C for 2 min, then: 95°C for 30 sec, 44°C for 30 sec, and 70°C for 1 min for 30 cycles. Sequencing gels were run following standard procedures (Garcia et al. 1996) and the autoradiograms read by two different people to confirm the sequence. Some plasmids were sequenced using the forward and reverse primers of M13 and electrophoresed at least twice, from 4–12 h, to obtain unique sequences on either side of the microsatellite. All motifs with three or more repeats were counted as microsatellites (Meehan et al. 2003). These researchers reported amplification of 51 out of 93 polymorphic microsatellites that contained single or multiple motifs of less than six repeats each, greatly increasing the number of useful markers. To compare with microsatellites frequencies reported in previous studies, motifs with 5 or more repeats, and 10 or more repeats were also counted (Xu et al. 1999, Meehan et al. 2003).

To identify potential genes and proteins in the shrimp genome, sequence homology comparisons were performed using Blastn and Blastx run against the currently available GenBank sequences (GenBank flat file release 158, February 15, 2007) based on a cut-off E-value of [E()] < 0.005.

Animals Used in This Study

Wild *L. vannamei* juveniles ($n = 24$) originated from Oaxaca, Mexico (candidate-SPF Population 4) and adult females ($n = 24$) from Salinas, Ecuador. Cultured shrimp originated from two SPF families (#1.4 and #1.5) of Population 1 and consisted of two parents and 15 and 14 third-generation offspring each, respectively. Population 1 originated from Sinaloa, Mexico and has contributed to the development of the current "Kona" Line (also called Research Line, Reference Line, or TSV-susceptible

Line); High Growth Line, and TSV-resistant Line of the USMSFP (Xu et al. 2003a).

Microsatellite Amplification and Scoring

The Primer3 program (Rozen & Skaletsky 1996, Rozen & Skaletsky 1997), as well as visual editing, was used to design 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., Alameda, CA, or Integrated DNA Technology, Inc., Coraville, IA) and used to amplify alleles in DNA (100 ng) from wild and cultured shrimp. The forward and reverse oligonucleotide primer sequences are listed in Table 1.

Polymerase chain reaction (PCR) mixture (25 μL) generally contained 100 ng DNA, 7.5 ng of γ-^{32}P-ATP labeled reverse primer, 50 ng of forward primer, 2.0 mM Mg Cl_2, 0.2 mM of dNTPs, 2.5 units of *Taq* polymerase (Promega, WI), and 1× buffer. Thermal cycler (PTC-100, MJ Research, MA) profile was: 94°C for 3 min, followed by 21 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min and ran for 21 cycles (Wolfus et al. 1997). Polyacrylamide gel electrophoresis of amplified products was performed using standard laboratory procedures. Samples were run next to a known sequence (*B20*; Garcia et al. 1996) to provide estimates of allele sizes. Some primer sets did not amplify DNA at the 52°C annealing temperature and were tested by varying the concentrations of $MgCl_2$ in different annealing temperature conditions. Three markers (TUGAPv1-3.224, TUGAPv5-7.33, and TUGAPv7-9.17) were used at 44°C (after optimization of annealing temperature conditions) to examine genetic diversity in wild and cultured shrimp. A microsatellite was regarded as polymorphic when the frequency of the most common allele was equal to or less than 0.99 (Nei 1987). Theoretical or expected heterozygosity levels were calculated as in Nei and Roychoudhury (1974) to adjust for small sample size ($h_e = 1 - \Sigma p_i^2[2N/(2N-1)]$) where p_i is the ith allele frequency and N = sample size. Observed heterozygosity was calculated based on the number of heterozygotes in a population divided by the total number of individuals analyzed in that population.

Linkage Mapping

The polymorphism status of a marker was first examined using a small test panel consisting of DNA from eight offspring of the International Reference Mapping Family (IRMF), which is being used to develop a framework linkage map for *L. vannamei* (*ShrimpMap*) and eight parental broodstock of four Resource Mapping Families (RMF) being used for identifying candidate genes associated with resistance to TSV (Alcívar-Warren et al. 2002). Genotyping was performed using a ^{32}P-based assay (Meehan et al. 2003). The polymorphic markers were then genotyped with the entire IRMF panel using either the ^{32}P-based assay or a protocol modified from the user's manual of ABI PRISM 377 DNA sequencer (Alcivar-Warren et al. 2007a, Alcivar-Warren et al. 2007b). Briefly, PCR mixture consisted of 0.3 μM (20 ng) template DNA, 0.333 μM reverse primer (fluorescently labeled with 6-FAM, TET, or HEX), 0.333 μM forward primer, 0.125 mM dNTPs, 0.04 U/μL Taq polymerase (Promega), 2.5 mM MgCl2 and 1× buffer in a total of volume of 15 μL. The following PCR profile was used in a MJ Research thermocycler PTC-100: 95°C for 12 min. followed by 30 cycles of 94°C for 1 min., annealing temperature for 1 min., 72°C for 2 min. and ending with 72°C for 30 min. The amplified products were then multiplexed by combining HEX (3 μl), TET (2 μL) and 6-FAM (2 μL) PCR product. Three μl of loading mix (250 μL of deionized formamide, 50 μL of GeneScan-500, and 25 μL of loading buffer) and 1 μL of the multiplexed PCR product were combined and used in loading the gel. After the ABI run was complete, GeneScan® Analysis Software processes the gel image. This was also manually checked (binned) to make sure all lanes used in the gel lined up properly and the size standard was applied appropriately. Once the gel image was processed, the information was exported into GenoTyper® Software which assigns sizes to the amplified product in each lane based on the size standard used. All genotypic results were compiled in an excel sheet and checked manually for potential genotyping errors. The allele sizes of amplified products were confirmed by two different researchers. The allele data obtained from the IRMF panel was examined using CRIMAP software with a limits-of-detection (LOD) score of 5.0 to accurately

TABLE 1.

Summary of forward and reverse primer sequences, allele number, and size ranges obtained after genotyping wild and cultured *Litopenaeus vannamei* with three microsatellites.

		Number of Alleles				Size Range (bp)			
		Wild[a]		Cultured[b]		Wild		Cultured	
Locus ID	Forward (F) and Reverse (R) Primer Sequences	Oaxaca, Mexico	Salinas, Ecuador	Family 1.4	Family 1.5	Oaxaca, Mexico	Salinas, Ecuador	Family 1.4	Family 1.5
TUDGLv1-3.224	F: 5′-ACTAGTGGATCTGTCTATTC-3′ R: 5′-ATACCCACCCATGCATGTTAG-3′	25 (21)	21 (18)	4 (17)	4 (16)	137–224	157–234	163–199	168–225
TUDGLv5-7.33	F: 5′-TGCTAGAATGTCTTTCGAAG-3′ R: 5′GTCTGGGGAAATCTTTAATG-3′	NA	31 (24)	4 (17)	4 (16)	NA	90–186	121–182	129–183
TUDGLv7-9.17	F: 5′-ATGGTGAATATAAGGAAGCT-3′ R: 5′-TGTGATATGGTTTTTGGAG-3′	26 (20)	31 (24)	4 (17)	4 (16)	96–274	95–275	125–157	125–199

[a] Numbers in parenthesis are number of individuals scored. NA = not scored; amplification profiles contained many stuttering bands making it difficult to determine the actual allele sizes. Annealing temperature for all loci was 44°C and $MgCl_2$ concentration was 2mM.
[b] See materials and methods section for details on the origin of these families.

identify linkage groups and determine marker order (Alcivar-Warren et al. 2007a).

RESULTS AND DISCUSSION

Library Cloning and Characterization of Microsatellites

One hundred and thirty-four positive clones were identified after hybridization with di- and trinucleotide probes from ~1,400 recombinant colonies obtained from four sized-fractionated genomic libraries. The distribution of positive clones is shown in Table 2. Probes $(GTG)_5$ and $(CG)_{15}$ did not hybridize to any of the clones. The library with the largest inserts (700–900 bp) had the greatest percentage (18.4%) of positive clones when compared with the number of colonies screened for this library. Only 4% of the clones were positive in the 100–300 bp library and 8.0% and 9.2% were positive in the 300–500 and 500–700 bp library, respectively (Table 2).

Out of the 134 positive clones, 128 sequences were used for analysis, 83 of which contained microsatellite motifs of three or more repeats, 3 had no microsatellites, and 42 were either identical to each other, contained too many "N"s, could not be sequenced, or contained less than 50 nucleotides. Three of the 83 clones were sequenced from both ends, providing a total of 86 different sequences for further analysis. The GenBank accession numbers for the 86 sequences are AF006629-AF006631 and AY376912-AY376997 (Table 3). Overall, results indicated that only 5.9% (83/1400) of the positive clones actually contained microsatellite repeat motifs which is less than the 11.7% reported for another *L. vannamei* library (Meehan et al. 2003).

A total of 340 microsatellite arrays were present in the 86 sequences, consisting of 312 di-, 19 tri, 31 tetra-, 4 penta-, 4 hexa- and, 1 octanucleotide motifs; alone or in combination; with three or more repeats (Table 3). Most of the motifs ($n = 371$) consisted of three or more repeats, whereas 178 motifs had five or more repeats and 104 consisted of 10 or more repeats. The most abundant di-, tri, tetra-, penta-, and hexa-nucleotide motifs were CT, CCT and CTT, CATA, CTTCT, and GAG ATA. The octanucleotide $(CCCTCTCT)_3$ was also identified.

Out of the 312 di-nucleotides motifs identified in these libxraries, 43% were CT ($n = 147$), followed by GT with 33% ($n = 104$), AT with 16% ($n = 49$) and CG with 3.8% ($n = 12$). These results are similar to those reported for *L. vannamei* (Meehan et al. 2003) and hymenopteran species like the yellowsjacket wasp and humble bee (Thoren et al. 1995) but different from those published in *D. melanogaster* (Schug et al. 1998) and most vertebrate species that found GT microsatellites to be the most abundant, followed by CT (Weber 1990, Estoup et al. 1993, Brooker et al. 1994). Interestingly, the 100–300 bp library only had CT ($n = 19$) positive clones (Table 2).

The number ($n = 19$) of trinucleotides reported here is lower than the number ($n = 139$) identified in another *L. vannamei* library (Meehan et al. 2003). The high number of CTT ($n = 6$) and CCT ($n = 6$) repeats found in this study may be attributed to hybridization with CT probe. $(CTT)_n$ was also one of the first microsatellite motifs isolated from a RAPD (*B20*) marker in *L. vannamei* (Garcia et al. 1996).

Tetra-nucleotide repeats ($n = 31$) were more abundant than trinucleotide repeats ($n = 19$) in this *L. vannamei* library, contrary to results from Meehan et al. (2003) for the same species, but similar to findings in both human and murine species (Astolfi et al. 2001). Tetra-nucleotide motifs included CATA ($n = 7$), CTTT ($n = 6$), CGCA ($n = 6$), and GACA ($n = 3$) among others. The $CTTT_n$ repeat was also isolated as part of M1 microsatellite (RAPD *B20* locus), a highly polymorphic marker that has been used to study genetic diversity of wild and cultured shrimp and track the pedigree of the USMSFP breeding program (Garcia et al. 1996, Wolfus et al. 1997) and examine allele frequency differences in TSV-resistant and TSV-susceptible shrimp (Xu et al. 2003a).

There were only 4 penta-nucleotide repeats in this library (2 CTTCT, 1 CCTTT and 1 AACCT), which was lower than the 35 reported for *L. vannamei* after probe hybridization of another ovarian genomic library (Meehan et al. 2003). Direct sequencing of all the clones obtained from the Meehan et al. (2003) library identified a large number of AACCT repeats in *L. vannamei* genome (Alcivar-Warren et al. 2002, 2006b and unpublished data). These pentanucleotide repeats appear to be the telomere sequences at ends of *L. vannamei* chromosomes and are also the site of introgression of telomere-specific retrotransposons in some insect and other arthropod species (Alcivar-Warren et al. 2006a). Similar AACCT repeats were reported by Bagshaw and Bucholt (1997). No AACCT repeats have been identified in *P. monodon* genomic libraries (Xu et al. 1999, Tassanakajon et al. 1998, Wuthisuthimethavee et al. 2003a).

Length and Frequency of Shrimp Microsatellites

Many of the shrimp microsatellites reported here contained three or more repeat motifs, but some contained up to 51 uninterrupted repeats. Indeed, 41 (47.7%) of the 86 sequences contained 25 or more uninterrupted repeats and 13 sequences contained 45 or more uninterrupted repeats, which differs from

TABLE 2.

Summary of positive recombinant clones identified in size-fractionated genomic libraries of *Litopenaeus vannamei* after hybridization with various oligonucleotide probes.

Library (bp)	# of Colonies Screened	# Positives with $(GT)_{15}$	# Positives with $(CT)_{15}$	# Positives with $(AT)_{15}$	# Positives with M_2	Total # of Positive Clones
100–300	415	0	19	0	0	19
300–500	410	9	24	0	0	33
500–700	325	17	10	1	2	30
700–900	250	21	19	0	6	46
Total	1400	47	72	1	8	128

TABLE 3.

Polymorphism status of microsatellite repeat motifs identified in 86 clones isolated from ovary genomic libraries of *Litopenaeus vannamei*.

Clone ID[a]	Forward and Reverse Primers (5′ → 3′)	*Repeat Motifs*[b]	Expected Size (bp)	Anneal. Temp (°F)	P[e] (# of alleles)[f]	Linkage Group in *ShrimpMap*	GenBank Accession #
TUGAPv 1-3.6	F: TACTNTCCACGCCACACTAA R: GATTGAGGGATTTTGATGGG	…**(TC)$_{25}$**…**(TC)$_{5}$**…	114	52	P (13)		AY376912
TUGAPv1-3.19F[c]		…(AG)$_{11}$AA(AG)$_{42}$			NT		AY376913
TUGAPv1-3.24F[c]		(AG)$_{51}$(A)$_{8}$(AG)$_{2}$CA(G)$_{12}$…			NT		AY376914
TUGAPv 1-3.49[d]	F: CACAGAAACACGCACACAAA R: ATTTGTGTGTGTGTGCAGAG	…**(CA)$_{3}$**…**(TC)$_{12}$**…**(TC)$_{5}$**…**(TC)$_{3}$**… **(TC)$_{10}$A(CT)$_{3}$G(CA)$_{6}$**…(CATA)$_{3}$…	105	52	N		AY376915
TUGAPv 1-3.66[d]	F: GTGGGATATATTGGT R: CGTGTGTGTAAAGAA	…**(AT)$_{3}$**…**(TC)$_{16}$**…**(CT)$_{6}$**…**(TC)$_{3}$**…**(AC)$_{4}$**…	85	52	N		AY376916
TUGAPv1-3.132[d]	F: CCGCCATCATCATCAACA R: TCATTCGGGTTCGAGACTC	…(GA)$_{3}$…**(CAT)$_{3}$**….**(TC)$_{3}$GG(TC)$_{25}$**…	118	52	P (8)	LG6	AY376917
TUGAPv1-3.184[c,d]	F: CATAACTTAGAATGTAAAAGAG R: AAGAAAATGGACAGGGCAGTT	…**(AG)$_{51}$**…**(CCTA)$_{3}$**…(CT)$_{3}$	249	52	N		AY376918
TUGAPv1-3.185[c]		(GACA)$_{3}$…(AG)$_{4}$…(CT)$_{29}$T(TC)$_{13}$TA(TC)$_{3}$… (TC)$_{3}$…(CCCTCTCT)$_{3}$CC(CT)$_{7}$…(CT)$_{3}$…			NT		AY376919
TUGAPv1-3.219	F: CAGGCAAGGTAACAGGCATT R: AATTCGTACATTTTA	…**(AG)$_{34}$**…**(AG)$_{4}$**…	276	52	NA		AY376920
TUDGLv1-3.224[d]	F: ACTAGTGGATCTGTCTATTCAT R: ATACCCACCCATGCATGTTA	…**(TAGA)$_{3}$**…**(TAGA)$_{3}$**…**(ACAG)$_{4}$** **(AG)$_{21}$A(AG)$_{30}$**…	185	44	P (9)		AF006629
TUGAPv1-3.254[c,g]	F2: ACTAGTGGATCTTCGGTTGT R2: GGATAGACTCGACAAATGGA	…**(TG)$_{10}$**…**(TA)$_{3}$**…(AG)$_{6}$AA(AG)$_{38}$	109	42–50	P (2)		AY376921
TUGAPv1-3.267	F: TTACACCGATCTTGACAATCATAG R: AGGCAGGGAGTCCTGTGAAC	…**(CT)$_{3}$**…**(CT)$_{3}$**…	191	52	N		AY376922
TUGAPv1-3.319		…**(TC)$_{21}$CC(TC)$_{3}$**…(GC)3…			NT		AY376923
TUGAPv1-3.339		…**(GAGATA)$_{5}$GATA(GA)$_{21}$**…			NT		AY376924
TUGAPv1-3.371[c]		…(CT)$_{48}$			NT		AY376925
TUGAPv1-3.381	F: GGAATGAATGGATGTGGATTG R: AACAGGCCTACAAATTTCACG	…**(GA)$_{3}$**…(GA)$_{17}$CT (AG)$_{17}$…	212	52	N		AY376926
TUGAPv1-3 387F[c]		…(AG)$_{7}$…(AG)$_{4}$			NT		AY376927
TUGAPv.3-5.1B		(TA)$_{3}$…(TC)$_{15}$C(CT)$_{3}$…(TC)$_{3}$..(TC)$_{3}$…			NT		AY376928
TUGAPv3-5.34[c,d]	F: CACTGAGCCCAGCACCTC R: GAGGGTGGAAGAGGAGGC	…**(TCCC)$_{3}$**…**(CCCT)$_{4}$**…(TC)$_{37}$TN(TC)$_{4}$…(TC)$_{6}$	109	52	N		AY376929
TUGAPv3-5.82	F: TCGCTGTGATTTGTTTTGGA R: CGTTATGGATCTCGTGGCTT	…**(TC)$_{4}$**…**(TC)$_{3}$**…**(TC)$_{5}$(GC)$_{4}$**…**(CT)$_{8}$CG(CT)$_{3}$**… **(TC)$_{4}$**…**(CT)$_{9}$T(TC)$_{21}$C(CT)$_{6}$**…**(CT)$_{7}$**…	332	52	P (3)		AY376930
TUGAPv3-5.147F[d,h]	F2: TTGATAGATACGCGC R2: GTGTTCAGGATGTTTAG	…(AT)$_{3}$…**(CG)$_{3}$(CA)$_{8}$(CGCA)$_{10}$(CA)$_{10}$T(AC)$_{4}$**…	118	40–42	P (2)		AY376931
TUGAPv3-5.175	F: TATGTCTCCCTCTTTCTCCC R: GGGATCTTGATTTGTGGGTG	…**(CT)$_{4}$**…**(CT)$_{4}$TC(CT)$_{3}$**…**(CT)$_{3}$T(TC)$_{6}$**… **(CT)$_{3}$TT(TC)$_{3}$**…**(TC)$_{6}$**…**(CT)$_{3}$**…**(TC)$_{6}$**… **(CT)$_{3}$TC(CT)$_{4}$**…**(TC)$_{6}$**…**(CT)3TC(CT)$_{6}$**… **(CT)$_{3}$(TCC)$_{3}$**…(GA)$_{6}$…(GA)$_{5}$…	387	52	P (2)		AY376932

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TABLE 3.

continued

Clone ID[a]	Forward and Reverse Primers (5′ → 3′)	*Repeat Motifs*[b]	Expected Size (bp)	Anneal. Temp (°F)	P[e] (# of alleles)[f]	Linkage Group in *ShrimpMap*	GenBank Accession #
TUGAPv3-5.200[c,d]	F: AAACCTTTCTTGGCAGCG R: GAAAGTGCAAAGAGTGTG	$(TC)_3$…**$(TC)_6(TCCC)_5$**…**$(CT)_3$**…**$(TC)_{45}T(CA)_3$**…	186	52	P (2)		AY376933
TUGAPv3-5.213	F: CCCAGAACCATGTGATTGC R: GTGAAGGGGGAATTATCCA	…**$(ATC)_3$**…**$(TTA)_3$**…**$(GA)_{49}$**…	281	52	P (2)	LG5	AY376934
TUGAPv3-5.222[c]		$(TG)_3(GA)_3$…$(TA)_4$…$(GC)_4$…$(CA)_3$… $(CA)_{17}$…$(CA)_3$…$(CA)_3$…$(CA)_3$			NT		AY376935
TUGAPv3-5.235[d]	F: AGACAGATAGATAGAGAGAG R: CGTTCTGCTTACATATTTGG	…**$(AG)_4$**…**$(AG)_6$**…**$(AG)_4$**…	54	52	P (3)		AY376936
TUGAPv3-5 237F[c]		…$(A)_7T(A)_4G(A)_5T(AG)_{48}$			NT		AY376937
TUGAPv3-5.242[c,d,h]	F: TCGTTCCTCTTCGTCTTTCG R: CTATGTTCGGAGCCTAGCCA	$(CTTCT)_3$…**$(CTTT)_3$**…**$(CTC)_3$**…**$(CT)_3$**… **$(TCC)_3$**… **$(CCT)_5$**…	293	52	NA		AY376938
TUGAPv3-5.256F[c]		…$(GAAA)_3(GA)_{47}$			NT		AY376939
TUGAPv3-5.259[c,d]	F: TGAGGTATAGGCAC R: GCTTTGTTAGTGCA	…**$(CA)_{43}$**…**$(AC)_7CT(AC)_4$**…**$(AC)_4$**…$(AC)_3$	125	52	N		AY376940
TUGAPv3-5.271	F: CCACCCAACGTTTAAATAAC R: GTCAGAGGATTGTATGATGT	…$(GA)_{50}$…	141	52	P (8)	Unlinked	AY376941
TUGAPv3-5.273	F: GACACCAGCACAAATGCAAA R: AGAGCGTGTTTGTGTATGTGTC	…**$(AC)_4TAG(AC)_5$**…**$(CG)_3$**…**$(AC)_4G(CA)_{10}$**…	87	52	N		AY376942
TUGAPv3-5.289F[c]		…$(TA)_3$…$(AG)_{48}AC(AG)_8$			NT		AY376943
TUGAPv3-5.292	F: GAGTTAGGACTGCTGTGC R: TACCCATACATAGATACC	…**$(TG)_3$**…$(TG)_3$…$(CA)_{16}$…	89	52	P (2)		AY376944
TUGAPv3-5.312[d]	F: ATCCTCAAAGACCTCCAGGG R: CACACAAAAGCTTCCTCAATG	…**$(GA)_3$**…**$(TG)_3$**… $(AG)_{51}$…$(CA)_{35}(TA)_{26}$…	69	52	P (2)		AY376945
TUGAPv3-5.337	F: GATCTATCGGTGCATGTTCA R: ACATTTTTGATAGAA	…**$(TG)_5$**…**$(TC)_{32}$**…**$(CT)_{10}$**…	280	52	NA		AY376946
TUGAPv3-5.342[g]	F: TAACTGTCTCAAAGCGTGGC RA: ATGGGAGNGAGGGACATAAA	…**$(TC)_{25}$**…	54	52	N		AY376947
TUGAPv3-5.350		…$(TC)_3$…$(AC)_{7…}(CACG)_4(CA)_{29…}$ $(CA)_{21}T(AC)_{14…}$			NT		AY376948
TUGAPv3-5.356		…$(AG)_3C(GA)_3TA(GA)_2(GAGATA)_3(GATA)_5$ $(GA)_{28}AA(GA)_2GT(GA)_{10}G(GA)_{6…}$			NT		AY376949
TUGAPv3-5.378	F: TCGGAAGGTGTCTTTCCAAAC R: AGGAAACCTATCATCGCCGT	…**$(AC)_5$**…**$(GC)_3G(CA)_3$**…**$(CA)_5CG(CA)_3$**… **$(GCAC)_3(AC)_5$**…**$(CA)_4$**…**$(AC)_{12}$**…	186	52	P (8)		AY376950
TUGAPv3-5.384		…$(AC)_6$..$(AC)_5$..$AT(AC)_{12}$…$(C)_9$…			NT		AY376951
TUGAPv3-5.391	F: TTATTTGCTTGCCCCCCTCC R: CGGGGGATCATACATCAACATC	…$(GC)_3$…**$(TC)_{14}$**…	114	52	P (9)	Unlinked	AY376952

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TABLE 3.

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Clone ID[a]	Forward and Reverse Primers (5′ → 3′)	*Repeat Motifs*[b]	Expected Size (bp)	Anneal. Temp (°F)	P[e] (# of alleles)[f]	Linkage Group in *ShrimpMap*	GenBank Accession #
TUGAPv5-7.9A		…$(TG)_3$…$(CA)_9$…$(AT)_{29}(CA)_{21}(TA)_{26}$…			NT		AY376954
TUGAPv5-7.33 (TU5733R in GB)	F: TGCTAGAATGTCTTTCGAAG R: GTCTGGGGAAATCTTTAATG	…**$(AC)_{11}AT(AC)_{14}$**…**$(CA)_{3}$**…	126	44	P (3)		AF006630
TUGAPv5-7.36	F: CTCATTCCCCATTATTCCCA R: TCTGCGCTAAATTGGTGTCA	…**$(CT)_4$**…**$(CA)_3$**…**$(CA)_3$**…**$(CA)_3$**…**$(CA)_{23}$** **$CT(CA)_3CT(CA)_{45}$**…**$(AC)_4AT(AC)_6$**… **$(AC)_3$**…**$(AC)_4$**…	477	52	P (3)		AY376955
TUGAPv5-7.41	F: GTTTTCTATCATGAATTCCC R: GACATTGGGAACTTAACG	…$(TC)_3TT(TC)_3$…**$(TC)_3$**…**$(CCT)_3T(CTC)_3$**… **$(TG)_3$**…**$(GA)_{42}$**…**$(TA)_3$**…**$(GT)_3$**…	430	52	NA		AY376956
TUGAPv5-7.74	F: ACCACTAAAATAACC R: ATTCAAATTCAAAGA	…**$(CA)_3$**…**$(CT)_3$**…	275	52	NA		AY376958
TUGAPv5-7.166[c]	F: AGGCGCTTTGGAGCAAGT R: ACCGAGTTCGCATTCCAG	…**$(CCTTT)_3CTCT(TC)_3$**…**$(TC)_4$**… $(TC)_4T(TC)_3T(TC)_3TTT(TC)_3TTT(TC)_5$… $(TC)_{33}$…$(CTTCT)_4$	95	52	P (2)		AY376959
TUGAPv5-7.167[c]	F: TTAATGACATCAATAGTAGCCT R: TCAAATACGAAAGCAGAGGACA	**$(C)_{12}$**…$(CT)_5T(TC)_3$…$(TC)_4G(TC)_4$… $(TC)_5TT(TC)_3$…$(CT)_5T(TC)_3$…$(TC)_4G(TC)_5$… $(TC)_5TT(TC)_7TT(TC)_{34}$	91	52	NA		AY376960
TUGAPv5-7.178[c]	F: TCACCTCTTGATGTGAAGTTGG R: CATTGGATTTTATCGCTGGC	…$(TC)_{20}(TA)_{27}NAT(GT)_3$…$(GT)_3$…**$(GA)_3$**…	210	52	NA		AY376961
TUGAPv5-7.203	F: ATTTCCCTTTCCCTTACCCC R: TACAAGCAAAGGGTGGATGC	…**$(TA)_9$**…	258	52	N		AY376963
TUGAPv5-7.204		…$(C)_{11}$…$(GT)_3$…			NT		AY376964
TUGAPv5-7.221	F: AACTGATATCGAGAAACGAG R: AGTGGAGGAGGCCGGG	…**$(ACAG)_3$**…**$(AC)_{14}G(CA)_3$**…**$(AC)_5$**…**$(CA)_3$**… **$(TACA)_3(TA)_{25}TG(TA)_6TN(TA)_7(T)_9$**…	288	52	P (2)		AY376965
TUGAPv5-7.264F[c]		…$(AAT)_3$…$(TA)_3$…$(TA)_3$…$(TA)_{28}$…$(TG)_{35}$			NT		AY376966
TUGAPv5-7.266[c]		…$(GT)_3$…$(TG)_3CG(GT)_{45}$			NT		AY376967
TUGAPv5-7.277[c]		…$(CA)_{14}$…$(CG)_5(TG)_6$			NT		AY376968
TUGAPv5-7.284[d,i]	F2: TTGGTGTGTGCGTGAGTGT R2: GGTTTAACTTCTGTCAGACTGC	…**$(TG)_3$**…**$(GTGTGC)_4$**…$(AT)_4$…	28	52	P (8)		AY3766969
TUGAPv5-7.298F[c]		…$(GT)_5$…$(TG)_{22}$…$(GT)_3$…$(AT)_3$…$(AT)_{29}$			NT		AY376970
TUGAPv5-7.309A[c]	F: TTCAGCTTGACCTTCGACCT R: CATTACGCTGGCTATGGTGA	…$(TG)_3$…$(TG)_4$… $(TG)_{19}C(GT)_{12}A(TG)_{10}AG(TGTA)_3(AG)_{6}$… $(AG)_3T(GC)_9$…**$(A)_{16}$**…**$(GAA)_3$**…**$(C)_{14}$**	299	52	P (7)		AY376971
TUGAPv5-7.322	F: GGACGCTTTTTATTGTTGTCG R: TGGTCATTAGGAACGCATACA	…**$(TC)_9$**…**$(CT)_4$**…**$(CT)_3T(TC)_3$**…**$(TC)_8$**…**$(TC)_6$**… **$(CT)_3$**…**$(TC)_{45}TT(TC)_4$**…**$(TTC)_3$**…**$(TG)_3$**…$(T)_8$…	350	52	NA		AY376972
TUGALv7-9.17	F: ATGGTGAATATAAGGAAGCT R: TGTGATATGGTTTTTGGAG	…$(T)_{13}N(TA)_4$…**$(AC)_{10}$**…**$(AC)_{11}$**…	91	44	P (16)		AF006631

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TABLE 3.

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Clone ID[a]	Forward and Reverse Primers (5′ → 3′)	*Repeat Motifs*[b]	Expected Size (bp)	Anneal. Temp (°F)	P[e] (# of alleles)[f]	Linkage Group in *ShrimpMap*	GenBank Accession #
TUGAPv7-9.28	F: TCGTTCTCCATTGCATGACT R: GGGGATTTGAACGATAAAAAG	…$(CT)_3$…$(CT)_3$…**$(CA)_6CG(CA)_8$**… **$(CA)_{28}(TA)_{22}$**…	390	52	P (3)		AY376973
TUGAPv7-9.35	F: GAAAGGATTCTGCCTTCGAG R: AGCGGATAACCGAAGAAGC	…**$(TA)_3$**…**$(T)_{10}$**…**$(AT)_3$**…	340	52	P (2)		AY376974
TUGAPv7-9.35F[c]		…$(TG)_4$…$(TG)_4TA(TG)_7$…$(TG)_{37}$			NT		AY376975
TUGAPv7-9.52	F: GCCTCATATGTTGTATTAGGCA R: ATCTTCCACATATAGGATT	…$(AAT)_3$…**$(CA)_3$**…**$(GA)_3$**… **$(AC)_{27}A(AT)_{11}$**…**$(CA)_3$**…	431	52	NA		AY376976
TUGAPv7-9.59[c]	F: TGAATTCCGACAGTAGGGTTG R: CAATCTGTGATGTGGATGCC	…**$(AC)_9$**…**$(CA)_5C(CA)_{30}TA(CA)_3$**…**$(CA)_7$** **$TA(CA)_7(CGCA)_3$**…**$(CA)_{15}$**…**$(CA)_6$**… **$(CA)_{13}$**…**$(GA)_3$**…$(AT)_{38}$	353	52	P (4)		AY376977
TUGAPv7-9.94	F: TTTTCTCTTTCCACCTCGCA R: AACATATTGCGGACCAGACA	…**$(TC)_{56}$**…**$(TG)_5$**…	255	52	P (5)	Unlinked	AY376978
TUGAPv7-9.94F	F: GCGCTGTCCTGTTATGTGAAAG R: CACAGACAACCCGAAAGCTAAA	…**$(CA)_5$**…**$(CA)_{14}(CATA)_3(TA)_{27}$**…**$(TA)_3$**… **$(TA)_4$**…$(G)_{14}$…$(GA)_{15}$…$(GA)_3$…	231	52	P (5)		AY376979
TUGAPv7-9.95[c]	F: GATCCTGCGAGTCACTTTATCTC R: TTTATTGCGTATCCCAGAAGC	…**$(TC)_3$**…**$(AT)_3$**…**$(AG)_3$**…$(TC)_{13}$…$(AT)_{24}$ $G(TA)_5TG(TA)_4TG(TA)_4$…$(AT)_3$…$(AT)_{24}$	282	52	P (4)	LG14	AY376980
TUGAPv7-9.115	F: ACGAGAATGCTGTCCGAAGT R: TGTGCACGTTTGTATCTGATTG	…**$(AC)_8AT(AC)_4$**…	178	52	P (4)		AY376981
TUGAPv7-9.117		…$(AT)_3$…**$(CA)_9TA(CA)_3$**…			NT		AY376982
TUGAPv7-9.119	F: CATGACCTGCCTTTAATCCC R: AAAGACAAGGAACGAGCGAG	…**$(TC)_{15}$**…**$(CT)_8$**…**$(C)_{10}(CT)_5A(TC)_3$** **$TT(TC)_8A(CT)_9$**…**$(CT)_5$**…**$(TC)_4$**…**$(CT)_5$**… **$(CT)_3$**…$(TC)_3$…	339	52	P (4)		AY376983
TUGAPv7-9.132[c]	F: TCGGTTAAATAATGTATGGATATGT R: CATGATGCTAGTTTTGGAGGTG	…**$(TA)_3$**…$(AT)_3$…$(TC)_{39}$…$(CA)_5$	137	52	P (2)		AY376984
TUGAPv7-9.134	F: TCGTGTTTCATGTGTAGGCTG R: ATGAGATGATAAGGGAGTGAATG	…**$(TTA)_3$**…**$(CA)_3$**…**$(AACCT)_3$**…**$(TTATCA)_3$**…	433	52	N		AY376985
TUGAPv7-9.137	F: ATCTAATTGGCCTGATAGC R: AGGAATATTGTGCTGAAGAG	…**$(TTC)_3$**…$(CT)_4$…$(CT)_5A(TC)_3$…$(CT)_{20}$… $(CT)_5(CCCT)_3$…$(CT)_5AT(CT)_3$…$(CT)_7$… $(TC)_3$…$(CT)_3$…$(CT)_4$…$(CT)_3$…$(CT)_6$ $T(TC)_3CT(TCCC)_3(TC)_3$…	131	52	P (2)		AY376986
TUGAPv7-9.142	F: CTTCTGCTTTCGCCAAATTC R: CTGGTAACACCTCTCCCACC	…**$(CA)_3$**…$(CT)_5$…$(CT)_3T(TC)_4$…	229	52	M		AY376987
TUGAPv7-9.154	F: GCTAACAAAGCTGAGAGGAAGAG R: TGGTATAAACGCAACTCGTGTC	…**$(GC)_3$**…**$(GATA)_3$**…**$(AT)_4$**… **$(AT)_4A(CATA)_4$**…**$(TA)_6$**…**$(TA)_5T(GT)_6$** **$(GC)_3(GT)_{22}$**…**$(GA)_3$**…**$(AG)_3$**…**$(GA)_7$**…	400	52	NA		AY376988

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TABLE 3.

continued

Clone ID[a]	Forward and Reverse Primers (5′ → 3′)	*Repeat Motifs*[b]	Expected Size (bp)	Anneal. Temp (°F)	P[e] (# of alleles)[f]	Linkage Group in *ShrimpMap*	GenBank Accession #
TUGAPv7-9.166	F: TCTGACTTCACTTCGCTTCAG R: TCTACCGCGTTGATACTATGG	…**(CT)$_4$**…**(TC)$_3$**…(CA)$_{11}$CT(CA)$_{32}$(TA)$_{12}$…(AT)$_3$…(TA)$_6$…(TA)$_{17}$…(AC)$_6$AG(AC)$_4$…(CA)$_{22}$…(AT)$_5$…(CT)$_6$…	142	52	N		AY376989
TUGAPv 7-9.179	F: GCTGTCTGGCAGTCATTAA R: TGAGGAAAGGATGGCTGAA	…**(TC)$_{25}$**…	52	52	P (11)	LG13	AY376990
TUGAPv7-9.188	F: GGCTGTCTGGCAGTCATTAAC R: CCCTTCCCTTCTTCCTTCTA	…(CT)$_3$…**(TC)$_{11}$TT(TC)$_{13}$**…**(CCCT)$_3$**…**(GC) $_3$**…**(GA)$_3$**…**(GGA)$_3$**…	485	52	NA		AY376991
TUGAPv7-9.202	F: TTATGATTGTGCGAGGAACG R: AAAAGGGAAACGAAGGATGG	…**(CA) $_9$**…	268	52	NA		AY376992
TUGAPv7-9.202F[c]		…(CAT)$_3$…(GAA)$_3$…(AAG)$_4$…(AAG)$_3$…(TG)$_7$TA(TG)$_3$A(GT)$_6$(TG)$_{19}$A(GT)$_8$N(GT)$_5$			NT		AY376993
TUGAPv7-9.226	F: GTGGCAATCACAACACCAAA R: GGTCTGGTTTTCTGTACTGTGA	…**(AGAA)$_4$**…	33	52	P (4)	Unlinked	AY376994
TUGAPv7-9.234	F: GGGAAGTTCTATAGATGTACGATACG R: CCAAAAAGTGCAAGCAGATG	…**(CA)$_3$**…**(CACG)$_3$(CA)$_3$CG(CA)$_4$**…**(CACG)$_6$(CA)$_6$T(AC)$_{43}$AT(AC)$_3$**…**(CA)$_5$(TA)$_3$**…**(ATAC)$_3$**…**(CA)$_{13}$**…(TAAA)$_5$…	366	52	P (2)		AY376995
TUGAPv7-9.247[c]	F: TTCAGTTCATTTCCGAACCC R: ACGTGAGAGTGGCACTTGAG	…**(TA)$_{27}$**…**(TA)$_3$**…**(AT)$_3$**…(AAG)$_3$	234	52	P (4)		AY376996
TUGAPv7-9.250	F: CAGTTCATTTCCAACCCCC R: ATGTGCAGCTGATGACTTC	…**(TA)$_{18}$**…**(TA)$_3$**…**(TATG)$_3$(CA) $_3$** …**(GA) $_3$**…	386	52	NA		AY376997

[a] Nomenclature for microsatellites is as described in Meehan et al. (2003). [b] Different microsatellites within a clone are separated by (…). Motifs in bold indicate the repeats flanked by the primers selected for analysis. The 86 sequences originated from 83 clones isolated from genomic libraries cloned using ovary DNA from an adult female of SPF Population 1 of the USMSFP. Most clones were sequenced using the reverse M13 primers, clones ending with F indicate that the forward primer was used for sequencing. [c] No enough flanking sequence on either side of the repeat motifs, but primers may have been designed from a single or combined motifs within the sequence. [d] First or last microsaqtellite repeat was included in the primer. [e] P = Polymorphic, M = Monomorphic, NA = did not amplify; N = need further optimization of annealing temperature; NT = not tested. [f] Also amplified in broodstock of the USMSFP breeding program. [g, h, i] Also tested other primer sets; [i] Has another small microsatellite, both polymorphic. Markers in bold are the eight polymorphic markers genotyped with the entire mapping panel (IRMF) being used to construct the linkage map for shrimp, *ShrimpMap*. (Linkage analysis performed using CRIMAP with LOD score of 5.0).

the mostly short repeats reported previously in *L. vannamei* (Meehan et al. 2003). Microsatellite repeats longer than 47, 60, and, 33 have been identified in rainbow trout, Atlantic cod and Atlantic salmon, respectively (Slettan et al. 1993, Brooker et al. 1994). In mammals, however, the longest size class ranged from 23–30 repeats (Weber 1990, Table 1 in Brooker et al. 1994).

The overall frequency of *L. vannamei* microsatellites with three or more repeats was 1/1.35 (Table 4), similar to that reported by Meehan et al. (2003). Di-nucleotides with three or more repeats had the highest frequency in this library, followed by tetra-nucleotide microsatellites. Among the di-nucleotides with three or more repeats, the frequencies of CT, GT, AT, and CG were 1/3.30 kb, 1/4.66 kb, 1/9.88 kb, and 1/40.37 kb, respectively, as reported in Meehan et al. (2003). Similar frequencies for CT (1/2.5 kb) and GT (1/8 kb) were found in yellow jacket wasp (Thoren et al. 1995). In *D. melanogaster*, however, GT was the most abundant microsatellite in arrays of five or more repeats, followed by TA (Schug et al. 1998). The frequencies of CT and GT reported here for *L. vannamei* are higher than those found in *P. monodon* (Tassanakajon et al. 1998, Brooker et al. 2000). Tassanakajon et al. (1998) found a low frequency of $(GT)_n$, (1/93 kb), and $(CT)_n$ (1/164 kb) in microsatellites with six or more repeats. Brooker et al. (2000) also reported a low frequency of $(GT)_n$, (1/164 kb), and $(CT)_n$ (1/1,200 kb) in *P. monodon*. It is possible that CT is most abundant because of the number of clones that tested positive to the $(CT)_{15}$ probe. However, only one clone tested positive to $(AT)_{15}$ probe but there are 49 motifs being accounted for, suggesting caution when analyzing microsatellites obtained after probe hybridization.

Microsatellite Allelic Diversity in Wild and Cultured L. vannamei

Three microsatellites (TUDGLv1-3.224, TUDGLv5-7.33, and TUDGLv7-9.17) were used for genotyping in wild and cultured shrimp. In wild shrimp, these markers showed a large number of alleles for each locus, ranging from 21–31, as well as a large size range for each locus, from 90 bp to 275 bp (Table 1). In shrimp from Mexico and Ecuador, there were 25 and 21 alleles at locus TUDGLv1-3.224, and 26 and 31 alleles at locus TUDGLv7-9.17, respectively. The shrimp of Mexico was not scored for locus TUDGL5-7 33R because the amplification profiles contained many stuttering bands and it was difficult to determine the actual allele size, suggesting that this marker should not be used for population genetic analysis. The wild shrimp from Ecuador had 31 alleles at locus TUDGL5-7.33.

The highest frequency for any allele (excluding null alleles) was 0.14. However, this was only for one allele at the TUDGLv1-3.244 locus. All other alleles had frequencies of less than 0.10, the majority ranging from 0.02–0.04. Because of the low frequency, the probability of having a homozygote for any one allele is very small, therefore, individuals that showed only one allele were considered to be heterozygous for a null allele. These null alleles could occur if a mutation in one or both of the priming sites has arisen, as shown for *L. vannamei* using microsatellite M1 of *B20* locus (Wolfus et al. 1997). With this assumption, the observed heterozygosity was 100% for Mexican and Ecuadorian stocks. The expected heterozygosity levels were slightly lower or equal to the observed values (97% to 100%). Relatively high heterozygosities using microsatellites have also been reported in wild *P. monodon* of Australia, Thailand, and Philippines (Brooker et al. 2000, Supungul

TABLE 4.

The distribution and frequency of microsatellite repeat motifs with three or more nucleotide repeats from 86 sequences isolated from adult ovary genomic libraries of *Litopenaeus vannamei*.

	Di-nucleotides[a]		Tri-nucleotides		Tetra-nucleotides		Penta-nucleotides		Hexa-nucleotides		Octa-nucleotides		Total	
Repeat of Motifs	**#**	**Frequency (1/kb)**	**#**	**Frequency (1/kb)**	**#**	**Frequency (1/kb)**	**#**	**Frequency (1/kb)**	**#**	**Frequency (1/kb)**	**#**	**Frequency (1/kb)**	**#**	**Frequency (1/kb)**
Three or more repeats	312	1/1.55	19	1/25.49	31	1/15.63	4	1/121.10	4	1/121.10	1	1/484.40	371	1/1.31
Five or more repeats	170	1/2.85	2	1/242.20	5	1/96.88	0	—	0	—	0	—	177	1/2.74
Ten or more repeats	101	1/4.80	0	—	1	1/484.40	0	—	0		0	—	102	1/4.75
L. vannamei[b,c,d]	433	1/1.43	139	1/4.48	40	1/15.46	35	1/17.66	10	1/61.82	—	—	658[c]	1/0.94[c]

[a] The estimated frequency of microsatellites was obtained by dividing the estimated total length of the *P. vannamei* genomic library (484,400 base pairs = 1,400 × estimated average insert length of 346 bp) by the total number of repeats then divided by 1000.

[b] For comparison purposes. Taken from Meehan et al. (2003) for three or more repeats. [c] Includes one nano-nucleotide microsatellite. [d] Frequency estimated based on the total length (618,222 bp) of sequenced clones (1,479 clones × estimated average insert length of 418 bp).

et al. 2000, Xu et al. 2001), *P. schimitti* of Brazil (Maggioni et al. 2003) and *P. setiferus* of the United States (Ball & Chapman 2003). In wild *L. vannamei* from Mexico to Panama, however, observed heterozygosities ranged from 0.241–0.388 (Valle-Jimenez et al. 2004). The presence of stuttering bands with marker TUGAPv5-7.33 made it difficult to score the wild shrimp from Mexico. Considering potential null alleles for this and other markers, perhaps we should first test for inheritance pattern of shrimp microsatellites before using them in population genetics, relatedness/kinship, and traceability studies.

In cultured shrimp, high levels of allele diversity were found with the three microsatellites in the two SPF families studied, even though they are third-generation captive bred. Allele sizes ranged from 121 bp to 183 bp at locus TUDGLv5-7.33, 163–225 bp at locus TUDGLv1-3.224, and 125–199 bp at locus TUDGLv7-9.17 (Table 1). All parents and offspring were heterozygous giving a 100% observed heterozygosity for each locus. All parental alleles in the two families for each locus were inherited in a Mendelian fashion, with no pedigree error detected. Results indicate that at least two of the three microsatellites tested are useful for genetic diversity studies of wild shrimp populations, and all three microsatellites are useful as a managing tool to trace and maintain quality of the pedigree and estimate allele diversity among lines of the USMSFP breeding program. The inheritance of microsatellites developed for *L. vannamei* by Meehan et al. (2003) have also been reported in two other selectively bred families of *L. vannamei* maintained in a breeding program in China (Zhang & Xiang 2005). Other microsatellites have also been developed for pedigree tracing and genetic diversity analysis of *L. vannamei* from the USMSFP breeding program (Wolfus et al. 1997, Xu et al. 2003a, Steinberg et al. 2004, Alcivar-Warren et al. 2006*b*) and other breeding programs of *L. vannamei* and other penaeid species (Vonau et al. 1999, Moore et al. 1999, Sugaya et al. 2002, Cruz et al. 2004, Zhang & Xiang 2005).

There seems to be a very large size range for alleles at each locus in wild and cultured shrimp (Table 1). In wild shrimp, the largest allele size range differences between the largest and smallest allele sizes at locus TUGAPv7-9.17 were 178 bp and 180 bp for shrimp of Mexico and Ecuador, respectively. The smallest allele size differences at locus TUDGLv1-3.224 in wild shrimp of Mexico and Ecuador were 87 bp and 77 bp, respectively. In cultured shrimp, however, the allele size range differences at all three loci examined was smaller (32–74 bp) than in wild shrimp (77–180 bp). The larger size range differences observed in wild shrimp would occur if there were null alleles or a high mutation rate in these sequences. An average mutation rate of 5.0×10^{-3} with a 95% CI of 2.4×10^{-3}–7.6×10^{-3} has been reported for other *L. vannamei* microsatellites (Xu et al. 2003b), which falls within the range of 10^{-2} to 10^{-6} reported for other species. Results indicate that inheritance patterns, null alleles, and mutation rate of microsatellite markers should first be tested before using them on population genetic, kinship/individual relatedness, or traceability studies.

Microsatellite Polymorphism in Mapping Families

Fifty nine (71.1%) of the microsatellite loci had unique flanking sequences to design primers covering all the motifs included in the clones (Table 3). These results were similar to those found in *L. vannamei* (Meehan et al. 2003, Alcivar-Warren et al. 2006a), *P. monodon* (Xu et al. 1999), and *P. stylirostris* (Vonau et al. 1999) but different from results in *P. japonicus* (Moore et al. 1999) and *P. monodon* (Tassanakajon et al. 1998, Pongsomboon et al. 2000, Brooker et al. 2000).

In an effort to increase the number of polymorphic markers for mapping studies, 62 primer sets were designed that included single or multiple motifs with three or more repeats (Meehan et al. 2003). Thirty five (56.4%) of the 62 primers successfully amplified scorable, polymorphic bands in DNA from stocks of the reference and resource mapping families, with allele sizes ranging from 67 bp to 323 bp. The remaining primer sets were either monomorphic ($n = 1$) or did not amplify at the annealing temperature used or amplified many unscorable bands ($n = 26$). Further optimization of allele amplification conditions for the 26 clones listed as N/NA in Table 3 may increase the number of useful markers from this genomic library. These results differ from those of other researchers who have suggested limitations for using genomic microsatellite markers for genetic mapping and other genetic studies (reviewed in Meehan et al. 2003). The polymorphic markers reported here for *L. vannamei* add to the growing number of SSR markers isolated for this species from both genomic (Cruz et al. 2002, Meehan et al. 2003, Alcivar-Warren et al. 2006a, Jia et al. 2006, Freitas et al. 2007) and cDNA libraries (Van Wormhoudt & Sellos, 1996, Alcivar-Warren et al. 2003, Perez et al. 2005, Wang et al. 2005, Wuthisuthimethavee et al. 2003b; Maneeruttanarungroj et al. 2006, Dhar et al. 2007, Alcivar-Warren et al. 2007b), and will be useful to develop a high-density linkage map for penaeid shrimp species.

Linkage Analysis

Out of the 35 polymorphic markers, 14 were randomly selected for genotyping with the entire IRMF panel of *ShrimpMap* and 8 of these amplified in most of the individuals tested and were used for linkage analysis. CRIMAP analysis with LOD score of 3.0 placed three of the polymorphic markers (TUGAPv3-5.213, TUGAPv7-9.179, and TUGAPv7-9.95) on to linkage groups LG5, LG13, and LG14 of *ShrimpMap*, respectively, with five markers unlinked. Six markers did not amplify well in offspring of the IRMF panel and will be repeated. However, when linkage analysis was performed using CRIMAP with LOD score of 5.0 (Fig. 2 of Alcivar-Warren et al. 2007*a*), an additional marker (TUGAPv1-3.132) was placed in *ShrimpMap*'s LG6 and four markers (TUGAPv3-5.271, TUGAPv3-5.391; TUGAPv7-9.94; TUGAPv7-9.226) remained unlinked (Table 3, in bold).

Many of the 35 polymorphic markers developed from this library had null alleles, including four of the eight markers tested with the entire mapping panel and used for CRIMAP analysis [TUGAPv3-5.213 (LG5), TUGAPv3-5.271 (unlinked), TUGAPv7-9.94 (unlinked), and TUGAPv7-9.179 (LG13)], suggesting that markers with null alleles can be useful for linkage mapping. Current efforts focus on optimization of amplification conditions for other potential microsatellites developed from this and other genomic libraries to increase density of *ShrimpMap* and provide a more accurate estimate of the genome size of *L. vannamei* and other penaeid species. Considering the high cost of developing microsatellite markers, efforts should be directed to development of EST-SSRs and SNP markers for mapping.

Sequence Comparisons: non-LTR Retrotransposon Reverse Transcriptase and Other Genes

Homology sequence comparison using Blastn against the EST databases showed similarities to only the repeats present in ESTs of unknown function or known genes from other species. Homology searches using Blastx showed that 49 of our genomic clones were similar to hypothetical or unknown proteins of various organisms, 16 were similar to known genes (retinitis pigmentosa GTPase regulator, RNA binding motif protein 25, integrin beta-like, hydroxyproline-rich glycopreotein precursor, etc.), and 24 sequences had no homology to any sequence in the public database. Four of the markers placed on *ShrimpMap* (TUGAPv1-3.132, TUGAPv3-5.391, TUGAPv7-9.179, and TUGAPv7-9.226) had no homology to any sequence in the Genbank database and thus represent novel sequences in the shrimp genome.

Our clone TUGAPv7-9.28 (AY376973) showed partial homology (53-119 nt) to motifs of RNA-directed DNA polymerase (reverse transcriptase) gene from various species including *Leishmenia infantum* (XP_001465072) and *P. monodon* (ABB73282.1). Partial homology to similar motifs of *Leishmenia infantum* (XP_001465072) were found in eight additional clones from this library. Presence of reverse transcriptase gene is usually indicative of active transposable elements such as a non-LTR (long terminal repeat) retrotransposons. A large number of clones with similarities to motifs of non-LTR retrotransposon reverse transcriptase gene and other transposable elements were also identified in sequences from another genomic library cloned from ovary of SPF *L. vannamei* (Meehan et al. 2003, Alcivar-Warren et al. 2006a) and from a cDNA library of whole shrimp challenged with White Spot Syndrome Virus (Alcivar-Warren et al. 2007b). The first report of transposable elements in penaeid shrimp originated from 66 sequences of a genomic library of *P. monodon* (Xu et al. 2004). Using Blastn against nr databases, these researchers identified homologies to portions of four known genes (sbm and Rfe genes of *E.coli*, 18s rRNA, and O1GC3 sensory organ-specific membrane guanylyl cyclase) and three uncharacterized sequences, whereas sequence comparison against EST databases identified three genes (methymalonyl-COA mutase, 18s rRNA, and hemocyte-glutamine gamma-glutamyl transferase) and three uncharacterized sequences. Using Blastx, Xu et al. (2004) identified 1 hypothetical protein, 2 uncharacterized sequences, and 9 known homologs, 7 of which were similar to transposable elements of other species (three transposable-like elements from *Culex pipiens* and *Drosohpila*, a *sbm* protein metal binding cobalt from *E. coli*, two reverse transcriptase *Penelope*-related retrotransposons from *Schistosoma* and *Drosophila*, a lipopolysaccharide biosynthesis protein wzzE from *E. coli*, a non-LTR retrotransposon from *Schistosoma*, and a reverse transcriptase-like protein from *Bos taurus*). Results indicate that *in silico* data mining approach using sequences from genomic libraries of *L. vannamei* and *P. monodon* (this study and Xu et al. 2004), especially using Blastx, provide valuable information in identifying transposable elements and other expressed genes in shrimp that would be useful for shrimp linkage and comparative mapping studies.

In summary, results demonstrate that useful microsatellite genetic markers can be obtained from *Sau*3 A-digested genomic libraries of *L. vannamei*, some of which are useful for population genetic analysis and pedigree tracing in breeding programs. They also provide an abundance of variation from which to develop a high-density linkage map for shrimp. Moreover, some of the polymorphic markers had significant homology to various hypothetical and known proteins in the GeneBank database and suggest that sequences from a genomic library can also provide valuable information in identifying functional markers in shrimp.

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