J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-016-2777-3
ISSN 1672-5182, 2016 15 (2): 311-317
http://www.ouc.edu.cn/xbywb/
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Development of Three Multiplex PCR Primer Sets for Ark Shell (*Scapharca broughtonii*) and Their Validation in Parentage Assignment

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(Received October 17, 2014; revised December 19, 2014; accepted December 10, 2015)

Abstract *Scapharca broughtonii* is a commercially important and over-exploited species. In order to investigate its genetic diversity and population structure, 43 novel polymorphic microsatellites were isolated and characterized. The number of alleles per locus ranged from 3 to 22 with an average of 6.93, and the observed and expected heterozygosities varied between 0.233 and 1.000, and 0.250 and 0.953, with an average of 0.614 and 0.707, respectively. Three highly informative multiplex PCRs were developed from nine of those microsatellites for *S. broughtonii*. We evaluated and validated these multiplex PCRs in 8 full-sib families. The average polymorphism information content (PIC) was 0.539. The frequency of null alleles was estimated as 3.13% of all the alleles segregation based on a within-family analysis of Mendelian segregation patterns. Parentage analysis of real offspring demonstrated that 100% of all offspring were unambiguously allocated to a pair of parents based on 3 multiplex sets. Those 43 microsatellite loci with high variability will be helpful for the analysis of population genetics and conservation of wild stock of *S. broughtonii*. The 3 sets of multiplex PCRs could be an important tool of pedigree reconstruction, population genetic analysis and brood stock management.

Key words *Scapharca broughtonii*; microsatellites; multiplex PCR; parentage assignment

1 Introduction

The ark shell (*Scapharca broughtonii*) belongs to phylum Mollusca, class Bivalvia, order Arcoida, family Arcoidea, genus *Arca*, which is widely distributed along the northwestern Pacific coast, especially in China, Japan and South Korea. Owing to its high nutritive and economic values, *S. broughtonii* is one of the most commercially important species in these countries and has been cultured for many years (Liu *et al*., 2013). In recent years, however, the wild stock of *S. broughtonii* has experienced dramatic population decline due to over-exploitation and the deterioration of coastal environment (Li and Li, 2008). The decline of *S. broughtonii* stock causes people to pay close attention to its genetic variation and population structure which will provide essential information for the maintenance and management of clam genetic resources.

Microsatellite or simple sequence repeat (SSR) markers are co-dominant, multiallelic and highly polymorphic, thus have been widely used in population genetics and conservation of biological resources. To date, about 106 microsatellite markers have been developed for *S. broughtonii* (An and Park, 2005; Li and Li, 2008; Sekino *et al*., 2010; Li *et al*., 2012; Tian *et al*., 2012), including 81 genomic SSRs and 25 expressed sequence tag derived SSRs (EST-SSRs). These markers provide sufficient information to evaluate wild and cultured genetic resources, but are still deficient for the conservation program of this species. Consequently, many more loci should be developed for population genetics and construction of a genome map which will be of great benefit to related studies and protecting measures.

Microsatellites amplified by polymerase chain reaction (PCR) as a single locus is time-consuming and expensive. Multiplex PCR should aid to reducing the time and cost associated with microsatellite genetic assays (Neff *et al*., 2000; Ezaz *et al*., 2004). Moreover, multiplex PCR decreases the repeated manipulation of a large number of samples and, therefore, the risk of handling errors (Porta *et al*., 2006).

In this study, we developed and characterized novel polymorphic microsatellites for *S. broughtonii*. In addition, we selected 3 sets of multiplex PCR primers from these new microsatellites. These works will provide a tool for the parentage assignment as was validated in 8 single-pair mating families.

2 Materials and Methods

2.1 Development and Characterization of Microsatellites

Genomic DNA was isolated with a modified phenol-

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chloroform protocol (Li *et al*., 2006) from foot muscle of a *S. broughtonii* caught from Qingdao, Shandong, China, and subsequently digested with *Mbo* I. DNA fragments were ligated to oligonucleotide adapters (Yuan *et al*., 2009). Size fractions of 400–1000 bp were isolated and hybridized to Biotin-labeled dinucleotide repeat sequences [$(CA)_{15}$ and $(CT)_{15}$] after electrophoresis on a 2% NuSieve GTG agarose gel. Then the hybridization complex was lifted out with streptavidin-coated magnetic spheres (Promega). After washing, the SSR enriched DNA was eluted from the beads and amplified. The selected fragments were ligated to pMD19-T vector (Takara) and then transformed into *E. coli* DH5α competent cells (Takara). The white clones were picked out through blue/white screening, and verified with 2 vector primers and non-biotin-labeled $(CA)_{12}$ and $(CT)_{12}$ primers (Yuan *et al*., 2009). Screening amplifications were performed as follows: 94℃ for 3 min followed by 35 cycles of 94℃ for 45 s, 56℃ for 45 s and 72℃ for 45 s, then a final extension at 72℃ for 5 min. In total, 184 clones that generated two or more bands were chosen and sequenced using BigDye Terminator Cycle sequencing kit and an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). A total of 134 sequences that contained microsatellites with at least four uninterrupted repeats were screened by the software of SSRHunter1.3 (Li and Wan, 2005). After abandoning hybrid clones, duplicates and those with short unique regions flanking the microsatellite array, 85 sequences were found suitable for primer design. PCR primers for each microsatellite loci were designed by using Primer Premier 6.22 (http://www.premierbiosoft.com/), and tested on 30 natural individuals of *S. broughtonii* captured from Rizhao, Shandong, China. PCR reaction was performed in a 10 μmol L^{-1} volume containing 0.25 U *Taq* DNA polymerase (Takara), 1×PCR buffer, 0.2 mmol L^{-1} dNTP mix, 1 μmol L^{-1} of each primer set, 1.5 mmol L^{-1} $MgCl_2$ and about 100 ng template DNA. The conditions were as follows: 3 min at 94℃ followed by 35 cycles of 45 s at 94℃, 45 s at the annealing temperature listed in Table 1 and 45 s at 72℃, then a final extension of 5 min at 72℃. The PCR product was resolved by 6% denaturing polyacrylamide gel and silver staining. A 10-bp DNA ladder (Invitrogen) was used as length references of allele size. The number of alleles and observed (H_O) and expected (H_E) heterozygosities were estimated by Microsatellite Analyzer (MSA) software (Dieringer and Schlötterer, 2003). Tests for linkage disequilibrium (LD) and deviations from Hardy-Weinberg equilibrium (HWE) were performed by GENEPOP 4.0 (Rousset, 2008). Null alleles, stuttering and large allele dropout were analyzed by Micro-Checker software (Van Oosterhout *et al*., 2004). All results for multiple tests were corrected using a Bonferroni's correction (Rice, 1989).

2.2 Multiplex PCR and Microsatellite Genotyping

All the microsatellite primers were chosen as the candidates of multiplex PCR according to their polymorphism, amplification efficiency and scoring clarity. The best 9 microsatellites were selected for multiplex PCR. The selection criteria were as follows: good amplification product yield, high degree of polymorphism, little or no PCR artifact arising from nonspecific amplification and ease of allele determination.

Eight *S. broughtonii* single-pair mating families were produced in late May, 2013 from adult blood clams obtained along the coast of Weihai, Shandong, China. DNA from 30 larvae each family was prepared with Chelex extraction method (Li *et al*., 2003), and DNA from both parents was extracted from muscle with the same method. These 9 loci were grouped into multiplex PCR sets that maximize the number of loci suitable for simultaneous amplification with no allele overlap between loci. Primer concentration and annealing temperature were then optimized using 6 individuals. Multiplex PCR was conducted in a 10 μL reaction volume containing 1× PCR buffer, 0.25 mmol L^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$, 0.5 units of *Taq* DNA polymerase (Takara), 0.3 μmol L^{-1} each of forward and reverse primers and 1 μL genomic DNA.

2.3 Genetic Analysis and Simulation Analysis

The polymorphic information content (PIC) and the average non-exclusion probability each loci in different situations were calculated using Cervus 3.0 (Kalinowski *et al*., 2007). In order to test the general resolving power of the multiplex PCR, we pooled the genotype data of the progeny in all the 8 single-pair families. The allele frequencies of 16 parents and 240 progeny were used to running the simulation based on the following parameters: 10000 replication cycles, a pool of 16 candidate parents, 100% of the candidate parents sampled and genotyped. To ascertain if alleles are inherited in a Mendelian fashion, all genotypic ratios were tested against the expected Mendelian segregation ratios (1:1, 1:2:1, and 1:1:1:1) using χ^2 analysis (with n-1 degrees of freedom, n is the number of marker-phenotypic classes) at 0.01 probability level (Li *et al*., 2010).

3 Results and Discussion

In total, 43 microsatellite loci were found polymorphic (Table 1). The number of alleles per locus ranged from 3 to 22 with an average of 6.93, and the observed and expected heterozygosities ranged 0.233–1.000 and 0.250–0.953, with an average of 0.614 and 0.707, respectively. Tests for linkage disequilibrium showed a nonrandom association ($P < 0.01$) between two pairs of loci (Sb43–Sb51 and Sb62–Sb69). Eighteen loci (Sb03, Sb04, Sb06, Sb26, Sb28, Sb32, Sb34, Sb36, Sb42, Sb47, Sb48, Sb51, Sb55, Sb61, Sb62, Sb65, Sb66 and Sb67) deviated significantly from HWE after Bonferroni's correction, which may be caused by the limited sample size and the presence of null alleles (Pemberton *et al*., 1995). But no stuttering and large allele dropout were found in all markers.

Nine microsatellites were allocated into 3 optimized multiplex PCR primer sets, each of which contains 3 markers. The annealing temperature of the whole multi-

plex primer sets and the concentrations of 3 pairs of primers are 2 key factors to organize a successful multiplex PCR. For each multiplex PCR, the suitable annealing temperature was ±6℃ around the T_a of the 3 markers (Table 1). The marker-specific variability and non-exclusion probability of each microsatellite are shown in Table 2. The PIC ranged from 0.267 to 0.727 with an average of 0.539, from which a high exclusion power was revealed for these multiplex PCR in parentage analysis. Overall, the genetic parameters for these multiplex PCRs are not convincing, which were satisfying in the development and characterization of microsatellites (Table 1). This may be due to the presence of null alleles and the close genetic relationship of the parents.

Table 1 Level of variability at 43 polymorphic microsatellite loci in *S. broughtonii*

Locus	GenBank accession no.	Repeat motif	Primer sequence (5'-3')	T_a (℃)	No. of alleles	Size (bp)	H_o	H_E	*P*-value
Sb01	KF203135	$(TG)_{14}GTGA(GT)_{14}N_{37}(TG)_{19}$	F:CATGCGTGTTTGTTCGTATAT R:ATACAGACATTGACATACACCAT	40	9	180-202	0.960	0.856	0.0066
Sb03	KF203136	$(TG)_9N_{35}(GT)_{10}$	F:GATCACAAGGAGTCACGGC R:CTACTTCTCCCTACCCTCT	54	4	226-232	0.650	0.755	0.0002*
Sb04	KF203137	$(TG)_6TA(TG)_5TA(TG)_5N_6(TG)_4$	F:GACAGGTCTGGGCTAACGAG R:GTTCACCCGCCACGACTC	42	4	264-278	0.500	0.753	0.0004*
Sb05	KF203138	$(CA)_4T(AC)_6(AT)_6$	F:GAAGCGGTGGTGGGAGTG R:ATGTAGTTTGGGGTTCAGTC	48	4	162-172	0.692	0.714	0.0036
Sb06	KF203139	$(CA)_4T(AC)_4N_6(AC)_6AT(AC)_5AT(AC)_6$	F:ACACGCCTCCGACAATCA R:GACAGGTCTGGGCTAACG	58	6	280-292	0.421	0.785	0.0003*
Sb10	KF203140	$(GT)_{29}$	F:AGGTCGGGATGGGTGGGT R:CACAGACGCACAAACATG	48	5	240-320	0.556	0.627	0.3029
Sb13	KF203141	$(TG)_{74}$	F:AGTTTTCAAAATTACCCTC R:GCTATGACCATGATTACGC	42	7	294-322	0.667	0.769	0.2394
Sb14	KF203142	$(CA)_7$	F:TACATATCAGTTAAGCAAGC R:ATGGATGGCAATACCAAAG	54	6	96-118	0.828	0.653	0.0418
Sb15	KF203143	$(TG)_{11}$	F:GCCTGAATGGCAAAACCTG R:GTGTGGAATTGTGAGCGGATA	58	7	300-342	0.538	0.669	0.4692
Sb17	KF203144	$(TG)_{14}$	F:AGAGGGGACACTTCAGTTT R:GCTATGACCATGATTACGC	42	4	184-206	0.269	0.424	0.0066
Sb19	KF203145	$(GT)_5N_{58}(GT)_5N_{36}(GT)_{11}$	F:ACGGCGATGTGAAGGGAA R:GTGTGGAATTGTGAGCGGATA	54	4	240-270	0.276	0.250	1.0000
Sb22	KF203146	$(AC)_{24}CGG(CT)_{29}$	F:GCGCCTAGTCCACTTGTA R:CTGCTTCCGTCTGGTTTG	40	5	280-300	0.480	0.611	0.0013
Sb25	KF203147	$(GT)_{21}$	F:CGAACTCCGATGTATGAAT R:CACAGGAAACAGCTATGACC	54	4	350-360	0.786	0.631	0.2118
Sb26	KF203148	$(GT)_7N_7(GT)_9N_7(GT)_{15}$	F:TGCTTCATAATAAAGGGTGG R:GTGGAATTGTGAGCGGATA	54	4	236-244	0.320	0.620	0.0003*
Sb27	KF203149	$(GT)_{23}$	F:AACATTTTCCAGATTTGA R:GCTATGACCATGATTACGC	48	5	242-276	0.893	0.748	0.0265
Sb28	KF203150	$(AC)_{49}T(CA)_{13}N_{32}(CA)_{11}$	F:GCACCTAGTCCACTTGTA R:ATACGATAGACAGAAGAGCAC	48	4	338-420	0.519	0.747	0.0003*
Sb32	KF203151	$(TG)_{16}N_{56}(TG)_{15}$	F:TCAGGGGAGTGAGACGGAT R:GTGTGGAATTGTGAGCGGATA	54	3	174-178	1.000	0.581	0.0000*
Sb33	KF203152	$(GT)_{12}$	F:GCCGAACTCCGATGTATGAA R:GCTATGACCATGATTACGCCAAG	56	3	176-184	0.583	0.494	0.7861
Sb34	KF203153	$(CA)_{18}(CT)_6N_{10}(TC)_4N_{10}(CA)_6$	F:AACCCAGTAAATATGACAC R:TTAGAAAGGCAAAATAGAAC	42	5	194-212	0.462	0.714	0.0002*
Sb36	KF203154	$(TG)_{15}$	F:CTGGGCAAAGGTGATGTA R:GTGGAATTGTGAGCGGATA	48	7	224-240	0.933	0.758	0.0009*
Sb37	KF203155	$(GT)_{38}$	F:AGCAGACCTGATACTGGGACA R:CGACCTATCTACTTGCCTATTTG	40	5	142-158	0.750	0.708	0.9140
Sb38	KF203156	$(GT)_4N_8(GT)_7GC(GT)_{11}$	F:TTGTCCAACGAGTATCTAAT R:CACACAGGAAACAGCTATGA	60	5	398-414	0.483	0.435	1.0000
Sb40	KF203157	$(AG)_{20}(GT)_5(GA)_5(GT)_5$	F:AGGGTGTTCTGGAAGGGT R:GTGTGGAATTGTGAGCGGAT	62	5	182-190	0.667	0.663	0.0275
Sb42	KF203158	$(TG)_{13}(GA)_{13}$	F:GTGAGGGTAAACAAAACG R:ATTTATGCCACAAGAACTAT	62	22	312-392	0.750	0.953	0.0000*
Sb43	KF203159	$(GA)_{15}$	F:CCAGTACCGTAACCTAACC R:GCAACTCAATCACATCCT	56	11	122-152	0.864	0.890	0.0018

(to be continued)

(continued)

Locus	GenBank accession no.	Repeat motif	Primer sequence (5'-3')	T_a (ºC)	No. of alleles	Size (bp)	H_o	H_E	*P*-value
Sb45	KF203160	$(AG)_{21}$	F:TCAGGGGTGGCAGAGGAA R:CAATGTTTTGATATTTTGCAGGTTT	64	10	202-222	0.679	0.896	0.0423
Sb47	KF203161	$(CA)_{20}(CT)_7(CA)_5(CT)_{20}$	F:AGTGTTCCAACCATCAAAT R:GTAATCAAGCACCTCCTGT	62	9	376-402	0.520	0.876	0.0000*
Sb48	KF203162	$(TG)_{16}N_{53}(TG)_{17}$	F:TTGTTAGATTTGTTTTGGTATGGG R:CGGCTCGTATGTTCTCACG	56	6	464-490	0.533	0.662	0.0001*
Sb51	KF203163	$(AG)_4N_{60}(GA)_{13}$	F:AAAATGGCATTCTAACACAT R:ACTAACAACCTGTAAATGAAATA	62	9	184-214	0.828	0.811	0.0008*
Sb52	KF203164	$(GA)_{15}(GT)_{24}$	F:ACCAGGGCGGCTAGGAAC R:TGTGTGGAATTGTGAGCGGAT	62	4	224-232	0.833	0.590	0.0262
Sb53	KF203165	$(GA)_{10}(GT)_{16}$	F:ATTTGTTTGTGCATGGGG R:GTGGAATTGTGAGCGGAT	48	5	202-212	0.667	0.565	0.1957
Sb54	KF203166	$(CT)_8N_5(TC)_6N_{21}(TC)_4N_{29}(CT)_6$	F:ACCCCATCATTCAACTGT R:GGTTTTATCCAGGCACTC	62	9	290-340	0.655	0.777	0.2519
Sb55	KF203167	$(GA)_{17}GCCT(GA)_{15}N_{46}(GA)_{12}$	F:GTTCTTCAACATAAACAGCGTG R:CGGTTCATGCCCTAATCA	62	14	316-448	0.708	0.909	0.0000*
Sb58	KF203168	$(GA)_6A(AG)_5N_{12}(GA)_{11}$	F:GATCCAGAGTGTCTTAGC R:CTATGACCATGATTACGC	42	6	432-446	0.800	0.750	0.0063
Sb60	KF203169	$(TC)_{12}$	F:CACATTGACTGACGACTTGGAT R:CCACTTACGGAGCGAGCA	58	8	102-122	0.536	0.594	0.2451
Sb61	KF203170	$(CT)_{11}CGCA(CG)_5(CT)_9T(TC)_{19}$	F:CACAAATGAGGTACAATGG R:AAACCGTGTCAGATGGAG	64	12	236-300	0.393	0.907	0.0000*
Sb62	KF203171	$(AG)_{17}$	F:ACAATAACACCGCCCCACC R:TGTCCGCTCGCAACAACT	66	9	164-188	0.269	0.839	0.0000*
Sb64	KF203172	$(AG)_{17}G(GA)_{14}N_{43}(GA)_{20}$	F:ACTGGAAACTCACAAAGG R:TGGTAGCACTGTAGTGGTT	40	7	254-298	0.625	0.607	0.3967
Sb65	KF203173	$(GT)_5(GA)_{14}N_{42}(TG)_{46}$	F:AGAGTGGCGACGACGAAAG R:TGTGGAATTGTGAGCGGATA	60	7	236-272	0.600	0.844	0.0005*
Sb66	KF203174	$(AC)_6T(CA)_4N_{13}(AG)_7N_{30}(GA)_5$	F:ATGCTCATCTACTAACAGTTAAT R:ACGAGACTGGATGCTGTA	64	9	288-350	0.233	0.476	0.0000*
Sb67	KF203175	$(TC)_{19}(CA)_{10}$	F:TCTACTACCACAGACCCTC R:TTAAAAGCCTTAACATAGC	62	11	310-344	0.519	0.875	0.0001*
Sb68	KF203176	$(CG)_4N_{39}(GA)_{15}$	F:CAATACAGCCAACCAAGC R:GCACCATCGGAAAATGAC	64	10	282-312	0.633	0.839	0.0146
Sb69	KF203177	$(CT)_7(TG)_4N_{60}(TG)_{10}T(TG)_{11}$	F:ACCGTAAGTCTTTAGGTG R:AGCGGATAACAATTTCAC	42	5	306-350	0.538	0.776	0.0338

Notes: T_a, annealing temperature; H_o, observed heterozygosity; H_E, expected heterozygosity; *, significantly deviated from Hardy-Weinberg equilibrium after sequential Bonferroni's correction ($P < 0.05/43$).

Table 2 Characteristics of the 3 multiplex PCR primer sets of *S. broughtonii*

Multiplex panel	Locus	GenBank accession no.	T_a (℃)	Size (bp)	Concentration in multiplex PCR ($\mu mol\,L^{-1}$)	N_a	H_o	H_e	PIC	NE-1P	NE-2P	NE-PP
Multiplex set 1	*Sb55*	KF203167	60	318-410	0.3	6	0.566	0.573	0.540	0.812	0.637	0.444
	Sb68	KF203176		282-308	0.3	5	0.625	0.724	0.676	0.694	0.520	0.340
	Sb62	KF203171		164-188	0.3	5	0.617	0.750	0.707	0.657	0.479	0.296
Multiplex set 2	*Sb54*	KF203166	60	306-340	0.3	5	0.496	0.766	0.727	0.635	0.455	0.272
	Sb61	KF203170		264-300	0.3	4	0.227	0.286	0.267	0.959	0.852	0.742
	Sb51	KF203163		192-208	0.3	5	0.602	0.646	0.597	0.763	0.595	0.410
Multiplex set 3	*Sb36*	KF203154	50	232-240	0.3	3	0.406	0.341	0.310	0.942	0.828	0.712
	Sb32	KF203151		174-178	0.3	3	0.672	0.543	0.456	0.853	0.738	0.606
	Sb43	KF203159		124-152	0.3	5	0.375	0.637	0.568	0.781	0.632	0.465

Notes: T_a, optimized annealing temperature of the primer; N_a, total number of alleles; PIC, polymorphic information content; NE-1P, average non-exclusion probability for one candidate parent; NE-2P, average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex; NE-PP, average non-exclusion probability for a candidate parent pair.

The results of Cervus simulations showed that the total assignment success was 65% using multiplex PCR primer set 1 alone, and was 100% with all 3 sets. The parentage analysis conducted in 8 single-pair mating families of *S. broughtonii* demonstrated that 71% of all offspring were correctly assigned to their parents with multiplex PCR primer set 1, and 100% of all offspring were unambiguously assigned to their parents with all 3 sets. This result

was comparable with those reported for *Crassostrea. gigas* (Li *et al*., 2010) and *Chlamys farreri* (Nie *et al*., 2012), where 3 multiplex PCR primer sets were used to successfully assign 100% of all offspring to a pair of parents. The number of loci required to assign parentage depends on several factors such as the overall information of the marker suite (which can be predicted in the mean PIC value), the combined exclusion power, the number of potential parents and the number of offspring to be assigned. The precision of assignment to correct parents depends not only on the number of microsatellites genotyped and their levels of polymorphism, but also on the number of potential pairings from which to choose (Norris *et al*., 2000). So in larger mating systems, more polymorphic multiplex primer sets are needed to achieve 100% assignment. However, the efficiency of parentage assignment will also be affected by many factors, such as the presence of null alleles, genotyping errors, mutations, non- Mendelian segregation and random allelic associations among loci (Castro *et al*., 2004; Vandeputte *et al*., 2011).

Table 3 reveals the parental genotypes, observed and expected genotypic ratios of offspring in each family at each of the three multiplex sets. Ten of the 72 genotypic ratios were monomorphic (AA × AA genotype), and thus resulted in offspring identical to the parents (Table 3). Of the 72 genotypic ratios examined, 22 genotypic ratios were still not compatible with Mendelian segregation after considering the presence of null alleles (Table 3). Departures from Mendelian ratios may be caused by several factors, such as zygotic viability selection, sampling or genotyping errors (Launey and Hedgecock, 2001). Zhan *et al*. (2009) reported that these departures from Mendelian segregation seem to be a common phenomenon in many marine molluscs. Otherwise, according to Launey and Hedgecock (2001), the lowest point of this segregation distortion is during the larvae stage due to the high genetic load with increasing selection afterwards. So conducting parentage analysis with larvae of marine molluscs is an effective and feasible way to decrease the segregation distortion. Nine of the 288 parental alleles among the nine loci were null alleles, which was 3.13% of the total alleles (9 loci × 16 parents × 2). The wide presence of null alleles is a classical source of error in parentage assignment with SSRs (Marshall *et al*., 1998), and will fail to apply these markers to population genetic analysis accurately. However, they may not affect the final result of parentage assignment.

Table 3 Genotypic proportions of microsatellite alleles in eight families of blood clam (*S. broughtonii)*

Family	Multiplex PCR primer set	Locus	Female	Male	Genotypes of progeny	Expected ratio	Observes ratio	χ^2	*P*
A	Multiplex set 1	*KF203167*	330/330	330/330	330/330	1	30	–	–
		KF203176	288/294	288/294	288/288:288/294:294/294	1:2:1	9:5:16	16.600	**0.000**
		KF203171	180/188	168/180	168/180:168/188:180/180:180/188	1:1:1:1	6:9:9:6	1.200	0.753
	Multiplex set 2	*KF203166*	306/314	306/314	306/306:306/314:314/314	1:2:1	16:8:6	13.200	**0.001**
		KF203170	300/null	280/300	(300/300+300/null):280/null:280/300	2:1:1	12:10:8	1.467	0.480
		KF203163	196/202	196/202	196/196:196/202:202/202	1:2:1	11:11:8	2.733	0.255
	Multiplex set 3	*KF203154*	238/238	232/238	232/238:238/238	1:1	22:8	6.533	0.011
		KF203151	176/178	176/178	176/176:176/178:178/178	1:2:1	5:10:15	10.000	**0.007**
		KF203159	124/124	136/152	124/136:124/152	1:1	11:19	2.133	0.144
B	Multiplex set 1	*KF203167*	330/360	330/360	330/330:330/360:360/360	1:2:1	10:11:9	2.200	0.333
		KF203176	300/308	300/308	300/300:300/308:308/308	1:2:1	8:6:16	15.067	**0.001**
		KF203171	168/null	180/188	168/180:168/188:180/null:188/null	1:1:1:1	8:7:8:7	0.133	0.988
	Multiplex set 2	*KF203166*	320/340	320/340	320/320:320/340:340/340	1:2:1	13:10:7	5.733	0.057
		KF203170	264/300	264/300	264/264:264/300:300/300	1:2:1	9:9:12	5.400	0.067
		KF203163	196/202	196/202	196/196:196/202:202/202	1:2:1	2:14:14	9.733	**0.008**
	Multiplex set 3	*KF203154*	232/238	238/238	232/238:238/238	1:1	13:17	0.533	0.465
		KF203151	176/178	176/178	176/176:176/178:178/178	1:2:1	0:30:0	30.000	**0.000**
		KF203159	124/152	124/150	124/124:124/152:124/150:150/152	1:1:1:1	0:12:14:4	17.467	**0.001**
C	Multiplex set 1	*KF203167*	330/null	330/360	(330/330+330/null):330/360:360/null	2:1:1	9:13:8	6.467	0.039
		KF203176	294/300	294/300	294/294:294/300:300/300	1:2:1	8:10:12	4.400	0.111
		KF203171	164/188	188/188	164/188:188/188	1:1	12:18	1.200	0.273
	Multiplex set 2	*KF203166*	320/330	320/330	320/320:320/330:330/330	1:2:1	10:3:17	22.467	**0.000**
		KF203170	300/300	280/300	280/300:300/300	1:1	14:16	0.133	0.715
		KF203163	202/null	196/202	(202/202+202/null):196/null:196/202	2:1:1	13:9:8	0.600	0.741
	Multiplex set 3	*KF203154*	238/238	238/238	238/238	1	30	–	–
		KF203151	176/176	176/176	176/176	1	30	–	–
		KF203159	124/124	124/124	124/124	1	30	–	–
D	Multiplex set 1	*KF203167*	330/340	330/410	330/330:330/410:330/340:340/410	1:1:1:1	12:2:10:6	7.867	0.049
		KF203176	300/308	288/294	288/300:288/308:294/300:294/308	1:1:1:1	5:9:6:10	2.267	0.519
		KF203171	176/180	180/180	176/180:180/180	1:1	13:17	0.533	0.469
	Multiplex set 2	*KF203166*	320/330	314/330	314/320:314/330:320/330:330/330	1:1:1:1	0:12:6:12	13.200	**0.004**
		KF203170	280/300	280/300	280/280:280/300:300/300	1:2:1	4:9:17	16.067	**0.000**
		KF203163	192/202	202/204	192/202:202/202:192/204:202/204	1:1:1:1	6:9:5:10	2.267	0.519

(to be continued)

(continued)

Family	Multiplex PCR primer set	Locus	Female	Male	Genotypes of progeny	Expected ratio	Observes ratio	χ^2	*P*
D	Multiplex set 3	*KF203154*	232/238	238/238	232/238:238/238	1:1	12:18	1.200	0.273
		KF203151	176/178	176/178	176/176:176/178:178/178	1:2:1	15:7:8	11.800	**0.003**
		KF203159	124/150	124/124	124/124:124/150	1:1	11:19	2.133	0.144
E	Multiplex set 1	*KF203167*	330/340	318/340	318/330:318/340:330/340:340/340	1:1:1:1	16:1:12:1	23.600	**0.000**
		KF203176	282/288	288/294	282/288:282/294:288/288:288/294	1:1:1:1	6:5:3:16	13.467	**0.004**
		KF203171	176/180	188/188	176/188:180/188	1:1	15:15	0.000	1.000
	Multiplex set 2	*KF203166*	330/340	320/330	320/330:320/340:330/330:330/340	1:1:1:1	7:4:10:9	2.800	0.424
		KF203170	300/300	300/300	300/300	1	30	–	–
		KF203163	196/204	202/204	196/202:202/204:196/204:204/204	1:1:1:1	13:15:2:0	23.067	**0.000**
	Multiplex set 3	*KF203154*	238/240	238/null	(238/238+238/null):240/null:238/240	2:1:1	13:10:7	1.133	0.567
		KF203151	176/178	174/176	174/176:174/178:176/176:176/178	1:1:1:1	9:0:1:20	34.267	**0.000**
		KF203159	136/null	124/136	(136/136+136/null):124/null:124/136	2:1:1	16:10:4	2.533	0.282
F	Multiplex set 1	*KF203167*	350/360	330/360	330/350:330/360:350/360:360/360	1:1:1:1	14:13:2:1	19.333	**0.000**
		KF203176	294/300	294/308	294/294:294/300:294/308:300/308	1:1:1:1	6:3:18:3	20.400	**0.000**
		KF203171	180/180	176/180	176/180:180/180	1:1	14:16	0.133	0.715
	Multiplex set 2	*KF203166*	330/340	306/320	306/330:306/340:320/330:320/340	1:1:1:1	4:3:11:12	8.667	0.034
		KF203170	300/300	300/300	300/300	1	30	–	–
		KF203163	196/202	202/208	196/202:202/202:196/208:202/208	1:1:1:1	11:9:2:8	6.000	0.112
	Multiplex set 3	*KF203154*	232/238	238/238	232/238:238/238	1:1	17:13	0.533	0.465
		KF203151	176/178	174/176	174/176:174/178:176/176:176/178	1:1:1:1	9:0:0:21	39.600	**0.000**
		KF203159	136/null	136/138	(136/136+136/null):138/null:136/138	2:1:1	12:11:7	2.267	0.322
G	Multiplex set 1	*KF203167*	340/360	330/410	330/340:330/360:340/410:360/410	1:1:1:1	8:8:9:5	1.200	0.753
		KF203176	294/308	308/308	294/308:308/308	1:1	20:10	3.333	0.068
		KF203171	164/168	164/168	164/164:164/168:168/168	1:2:1	7:10:13	5.733	0.057
	Multiplex set 2	*KF203166*	320/330	320/330	320/320:320/330:330/330	1:2:1	13:5:12	13.400	**0.001**
		KF203170	300/null	264/300	(300/300+300/null):264/null:264/300	2:1:1	13:7:10	1.133	0.567
		KF203163	202/208	196/202	196/202:196/208:202/202:202/208	1:1:1:1	6:11:9:4	3.867	1.633
	Multiplex set 3	*KF203154*	238/240	232/238	232/238:232/240:328/238:238/240	1:1:1:1	5:0:10:15	16.667	**0.001**
		KF203151	174/176	176/178	174/176:176/176:174/178:176/178	1:1:1:1	18:3:0:9	25.200	**0.000**
		KF203159	136/136	136/136	136/136	1	30	–	–
H	Multiplex set 1	*KF203167*	330/null	330/340	(330/330+330/null):340/null:330/340	2:1:1	15:12:3	5.400	0.067
		KF203176	294/308	294/300	294/294:294/308:294/300:300/308	1:1:1:1	7:13:7:3	6.800	0.079
		KF203171	180/188	164/188	164/180:164/188:180/188:188/188	1:1:1:1	8:5:5:12	4.400	0.221
	Multiplex set 2	*KF203166*	306/314	306/314	306/306:306/314:314/314	1:2:1	3:17:10	3.800	0.150
		KF203170	300/300	300/300	300/300	1	30	–	–
		KF203163	192/196	192/196	192/192:192/196:196/196	1:2:1	13:11:6	5.400	0.067
	Multiplex set 3	*KF203154*	238/238	238/238	238/238	1	30	–	–
		KF203151	176/178	176/178	176/176:176/178:178/178	1:2:1	5:25:0	15.000	**0.001**
		KF203159	124/124	124/124	124/124	1	30	–	–

Notes: Bolded *P*-values indicate genotypic ratios that do not conform to Mendelian segregation.

In summary, 43 microsatellite loci with high variability will be helpful for the analysis of population genetics and conservation of the wild stock of *S. broughtonii*. Additionally, 3 multiplex PCR assays were developed for the clam, and the results proved that the 3 multiplexed microsatellite systems can be applied to parentage assignment. The 3 sets of multiplex PCR primers could be an important tool of pedigree reconstruction, population genetic analysis, and brood stock management of *S. broughtonii*.

Acknowledgements

The study was supported by National Marine Public Welfare Research Program (201205023), National Natural Science Foundation of China (41276138), and Doctoral Program of Ministry of Education of China (20130132110009).

References

An, H. Y., and Park, J. Y., 2005. Ten new highly polymorphic microsatellite loci in the blood clam *Scapharca broughtonii*. *Molecular Ecology Notes*, **5**: 896-898.

Castro, J., Bouza, C., Presa, P., Pino-Querido, A., Riaza, A., Ferreiro, I., Sanchez, L., and Martinez, P., 2004. Potential sources of error in parentage assessment of turbot (*Scophthalmus maximus*) using microsatellite loci. *Aquaculture*, **242**: 119-135.

Dieringer, D., and Schlötterer, C., 2003. Microsatellite analyser (MSA): A platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, **3**: 167-169.

Ezaz, M. T., Sayeed, S., McAndrew, B. J., and Penman, D. J., 2004. Use of microsatellite loci and AFLP markers to verify gynogenesis and clonal lines in Nile tilapia *Oreochromis niloticus* L. *Aquaculture Resources*, **35**: 1472-1481.

Kalinowski, S. T., Taper, M. L., and Marshall, T. C., 2007. Re-

vising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, **16**: 1099-1106.

Launey, S., and Hedgecock, D., 2001. High genetic load in the Pacific oyster *Crassostrea gigas*. *Genetics*, **159**: 255-265.

Li, J. J., and Li, Q., 2008. Isolation and characterization of twelve novel microsatellite loci in the ark shell *Scapharca broughtonii*. *Conservation Genetics*, **9**: 1055-1057.

Li, M., Zhu, L., Zhou, C. Y., Lin, L., Fan, Y. J., and Zhuang, Z. M., 2012. Development and characterization of EST-SSR markers from *Scapharca broughtonii* and their transferability in *Scapharca subcrenata* and *Tegillarca granosa*. *Molecules*, **17**: 10716-10723.

Li, Q., Park, C., and Kijima, A., 2003. Allelic transmission of microsatellites and application to kinship analysis in newly hatched Pacific abalone larvae. *Fishery Science*, **69**: 883-889.

Li, Q., and Wan, J. M., 2005. SSRHUNTER: Development of a local searching software for SSR sites. *Hereditas*, **27**: 808-810.

Li, Q., Yu, H., and Yu, R. H., 2006. Genetic variability assessed by microsatellites in cultured population of the Pacific oyster (*Crassostrea gigas*) in China. *Aquaculture*, **259**: 95-102.

Li, R., Li, Q., Cornette, F., Dégremont, L., and Lapègue, S., 2010. Development of four EST-SSR multiplex PCRs in the Pacific oyster (*Crassostrea gigas*) and their validation in parentage assignment. *Aquaculture*, **310**: 234-239.

Liu, Y. G., Kurokawa, T., Sekino, M., Tanabe, T., and Watanabe, K., 2013. Complete mitochondrial DNA sequence of the ark shell *Scapharca broughtonii*: An ultra-large metazoan mitochondrial genome. *Comparative Biochemistry and Physiology D-Genomics & Proteomics*, **8**: 72-81.

Marshall, T. C., Slate, J., Kruuk, L. E. B., and Pemberton, J. M., 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**: 639-655.

Neff, B. D., Fu, P., and Gross, M. R., 2000. Microsatellite multiplexing in fish. *Transactions of the American Fisheries Society*, **129**: 584-593.

Nie, H. T., Li, Q., and Kong, L. F., 2012. Development of three multiplex PCRs in the Zhikong scallop (*Chlamys farreri*) and their validation in parentage assignment. *Biochemical Systematics and Ecology*, **44**: 96-101.

Norris, A. T., Bradley, D. G., and Cunningham, E. P., 2000. Parentage and relatedness determination in farmed Atlantic salmon (*Salmo salar*) using microsatellite markers. *Aquaculture*, **182**: 73-83.

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. *Molecular Ecology*, **4**: 249-252.

Porta, J., Porta, J. M., Martinez-Rotriguez, G., and Alvarez, M. D. C., 2006. Development of a microsatellite multiplex PCR for Senegalese sole (*Solea senegalensis*) and its application to broodstock management. *Aquaculture*, **56**: 159-166.

Rice, W. R., 1989. Analyzing tables of statistical tests. *Evolution*, **43**: 223-225.

Rousset, F., 2008. GENEPOP'007: A complete reimplementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, **8**: 103-106.

Sekino, M., Kurokawa, T., and Sasaki, K., 2010. Multiplex PCR panels of novel microsatellites for the ark shell *Scapharca broughtonii* (Pteriomorphia, Arcoida). *Conservation Genetics Resources*, **2**: 39-42.

Tian, J. T., Liu, Z. H., Zhou, L. Q., Wu, B., Liu, P., and Yang, A. G., 2012. Isolation and characterization of 48 polymorphic microsatellite markers for the blood clam *Scapharca broughtonii* (Arcidae). *Genetics and Molecular Research*, **11**: 4501-4507.

Van, O. C., Hutchinson, W. F., Wills, D. P. M., and Shipley, P., 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**: 535-538.

Vandeputte, M., Rossignol, M. N., and Pincent, C., 2011. From theory to practice: Empirical evaluation of the assignment power of marker sets for pedigree analysis in fish breeding. *Aquaculture*, **294**: 80-86.

Yuan, Y., Li, Q., Kong, L., and Pan, Y., 2009. Isolation and characterization of microsatellite loci in the tuba false fusus (*Hemifusus tuba* Gmelin). *Conservation Genetics Resources*, **1**: 9-11.

Zhan, A., Hu, J., Hu, X., Hui, M., Wang, M., Peng, W., Huang, X., Wang, S., Lu, W., Sun, C., and Bao, Z., 2009. Construction of microsatellite-based linkage maps and identification of size-related quantitative trait loci for Zhikong scallop (*Chlamys farreri*). *Animal Genetics*, **40**: 821-831.

(Edited by Qiu Yantao)