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Multiplex PCR Sets of Novel Microsatellite Loci for Iwagaki Oyster *Crassostrea nippona* and Their Application in Parentage Assignment

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Abstract Iwagaki oyster, *Crassostrea nippona*, widely distributes along the seashore of Eastern Asia, and has been considered to be a potential breeding species due to its delicious taste and high commercial value. In order to study its genetic background and population structure, we developed 46 novel polymorphic microsatellite markers using next-generation sequencing technique and characterized them in 30 individuals. The number of alleles ranged from 3 to 22, while the observed and expected heterozygosities varied from 0.133 to 1.000 and 0.455 to 0.949, respectively. Fifteen microsatellite markers were selected and grouped into five highly informative multiplex PCRs for *C. nippona*. We evaluated and validated these multiplex PCRs in a cultured population including 173 candidate parents and 486 offspring. In actual parentage analysis, 80% of the offspring were correctly assigned to their parental pairs using three multiplex PCRs. Furthermore, the success rate of parentage assignment reached 96% when the other two multiplex PCRs were added. These 46 microsatellite loci with high variability and the five multiplex PCRs described here provide a powerful tool for pedigree reconstruction, resource conservation and selective breeding program of *C. nippona*.

Key words *Crassostrea nippona*; microsatellites; multiplex PCR; parentage assignment

1 Introduction

Iwagaki oyster, *Crassostrea nippona*, is a marine bivalve belonging to Bivalvia, Ostreidae. It inhabits naturally along the coastal areas of Eastern Asia including Korea and Japan (Itoh *et al*., 2004; Yoon *et al*., 2008). The commercial value of *C. nippona* is high and its price is about five times of the price of Pacific oyster, because of its edibility in the summer when the other oyster species are not available in Japan (Itoh *et al*., 2004). Therefore, this species has broad market prospects and enjoys a great potential of aquiculture. Several hatcheries in China have gradually begun to introduce this species from Japan for artificial seedling and breeding in the recent years (Li, 2007; Yuan *et al*., 2008). Thus reasonable stock management and genetic improvement are becoming more and more important for sustainable development of *C. nippona* aquaculture industry. Molecular genetic markers are useful tools for genetic analysis and breeding in aquaculture. They play an essential role in stock identification, pedigree analysis, as well as genomic mapping (Liu and Cordes, 2004). Unfortunately, until now little information is available about the genetic background of *C. nippona*.

Microsatellites, also known as simple sequence repeats (SSRs), have been widely used in the study of population genetic structure and parentage determination due to their high polymorphism, co-dominance and abundance in the genome. However, microsatellite genotyping is costly and time-consuming. Multiplex polymerase chain reaction (PCR) is a technique that can amplify multiple sites simultaneously in the same reaction, thus saving a lot of time and money associated with microsatellite genetic studies. In addition, this technique decreases the risk of handling errors by reducing the repeated operation of a large number of experimental samples (Porta *et al*., 2006). Therefore, the development of multiplex PCR of microsatellite can provide powerful tools for large-scale family analysis, population genetic research and gene mapping studies. To date, multiplex PCR sets have successfully been developed and applied in many aquaculture species, including *Chlamys farreri* (Nie *et al*., 2012), *Crassostrea gigas* (Li *et al*., 2010; Liu *et al*., 2017) and *Scapharca broughtonii* (Li *et al*., 2016).

In the present research, we described the development and characterization of novel *C. nippona* polymorphic microsatellites as an important tool for future genetic re-

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search. Moreover, based on three primers PCR method (Blacket *et al*., 2012), we developed five sets of multiplex PCRs from these new microsatellites, and also tested the efficiency of these markers for parentage assignment in a cultured population.

2 Material and Methods

2.1 Sample Collection and DNA Extraction

Thirty *C. nippona* individuals used for microsatellites development were captured from Haiyi Aquaculture Cooperation, Yantai, China in 2017. Samples used for parentage assignment were also collected in late July, 2018 from Yantai. One hundred and seventy-three brood stocks (with unknown sexes) were selected for natural spawning. Four hundred and eighty-six D-larvae were collected at 48-h post-fertilization and then were preserved in 95% ethanol. For brood stock, genomic DNA was extracted from the adductor muscle using the phenol-chloroform procedure (Li *et al*., 2006). The extraction of genomic DNA from offspring was performed by the Chelex extraction method (Li *et al*., 2003). After extraction, genomic DNA of each sample was assessed by electrophoresis using 0.8 % agarose gel.

2.2 Development and Characterization of Microsatellites

To obtain microsatellite marker resources, we selected two individuals and performed Restriction-site Associated DNA sequencing (RAD-seq) using the Illumina HiSeq 2500 platform. DNA extraction, library preparation, amplification, and sequencing were carried out by Genedenovo (Guangzhou, China). The Primer3 software (Untergasser *et al*., 2012) was used to design the primers. One hundred and sixty primer pairs were randomly selected to test the polymorphism in thirty individuals of *C. nippona*. Amplification of each locus was performed in 10 μL reaction solution containing 0.5 U Taq DNA polymerase (Takara), 0.2 mmol L^{-1} of each dNTP, 1× PCR buffer, 2 mmol L^{-1} of $MgCl_2$, 1 μmol L^{-1} of each primer and about 50 ng template DNA. The conditions of PCR were as follows: initial denaturation at 94℃ for 3 min, followed by 32 cycles of denaturation at 94℃ for 30 s, annealing (annealing temperatures for different primer pairs are described in Table 1) for 45 s, and extension at 72℃ for 45 s per cycle, with a final extension at 72℃ for 5 min. After amplification, PCR products were separated by 6% denaturing polyacrylamide gels, and visualized by silver staining. A 10 bp ladder was used as a reference marker for allele size determination. For each microsatellite locus, the number of alleles (*Na*), observed (*Ho*) and expected heterozygosities (*He*), as well as the polymorphic information content (*PIC*) were calculated using the program CERVUS 3.0 (Kalinowski *et al*., 2007). Tests for Hardy-Weinberg equilibrium (HWE) were performed with GENEPOP 4.0 (Rousset, 2008).

2.3 Multiplex PCR and Genotyping

Based on the polymorphism of loci and the efficiency of amplification, the best fifteen microsatellites that showed clear PCR products were selected for multiplexing. Under the criteria of non-overlapping loci, these loci were allocated to multiplex sets and the number of loci suitable for simultaneous amplification was increased as many as possible. The universal primer M13-tail was labeled with different fluorescent dyes (VIC and FAM) so that the amplification products can be differentiated by capillary separation. At the same time, the proper primer concentration, annealing temperature and cycle times of each multiplex PCR were then optimized with 8 samples randomly selected from the above 30 individuals of *C. nippona*. Multiplex PCRs were performed in 10 μL reaction solution containing 0.25 U Taq DNA polymerase (Takara), 0.2 mmol L^{-1} of each dNTP, 1× PCR buffer, 2 mmol L^{-1} of $MgCl_2$, 0.06 or 0.08 μmol L^{-1} forward primer, 0.15 or 0.20 μmol L^{-1} universal primer, 0.15 or 0.20 μmol L^{-1} reverse primer, and about 50–100 ng template DNA. The conditions of PCR were performed as follows: initial denaturation at 94℃ for 3 min; followed by 35 cycles of denaturation at 94℃ for 30 s, 60 s at the optimal annealing temperature, and 72℃ for 75 s; then 8 cycles of denaturation at 94℃ for 30 s, 53℃ for 60 s, 72℃ for 75 s, with a final extension at 72℃ for 10 min. After amplification, PCR products were mixed with formamide and GeneScan 500-LIZ size standard (Applied BiosystemsTM Carlsbad, CA, USA) [0.5 μL PCR product, 9.5 μL Hi-Di Formamide (AppliedBiosystemsTM), 0.1 μL GS500-LIZ]. After 5 min of denaturation at 94℃, amplification products were rapidly cooled and detected by an ABI-3130xl Genetic Analyzer. Allele sizes were estimated with the program Gene-Mapper v4.0.

2.4 Simulation and Parentage Assignment

We used CERVUS 3.0 to calculate the *Na*, *Ho*, *He*, *PIC* and the non-exclusion probability of each microsatellite locus in this study. The genotypes of total 659 individuals, including 486 offspring and 173 candidate parents, were successfully analyzed using the optimized multiplex. The efficiency of the programin parentage assignment was validated. Similarly, the same program was also used to conduct the simulation and real parentage analysis based on the likelihood-based approach, and the parameters were as following: 10000 replication cycles, 50 to 300 candidate parents, 100% of the candidate parents sampled and genotyped, and strict 95% confidence level. We allowed 5% of typing errors in the assignment procedures, since this significantly reduces the impact of mismatches in parent-offspring relationships that may be caused by null alleles and mutations (Marshall *et al*., 1998).

2.5 Estimation of Effective Breeding Numbers (N_e)

The sibship reconstruction of the offspring and the inference of effective population size (N_e) were performed using COLONY 2.0 (Jones and Wang, 2010) without including parents. The analysis parameters were set as follows: diploid parents, both sexes polygamy, and 0.025 of the error rate of genotyping according to the suggestion of

Wang (2004).

3 Results

3.1 Properties of Microsatellite Markers

A total of 24811 SSR repeats from the assembled contigs were obtained in the study, among which dinucleotide was the most frequent (59.05%), followed by mononucleotide (17.60%) and tetranucleotides (13.36%) (Fig.1). In the study, we selected and designed 160 primer pairs at random. A total of 46 polymorphic microsatellite loci were amplified successfully, and the others were monomorphic or produced multi bands. The number of alleles ranged from 3 to 22 (Table 1), with the average of 12. The observed heterozygosity and expected heterozygosity varied from 0.133 to 1.000 and 0.445 to 0.949, respectively. The *PIC* of each locus had values between 0.399 and 0.929 with a mean of 0.780. Of the 46 polymorphic loci, 20 deviated significantly from HWE after a Bonferroni correction.

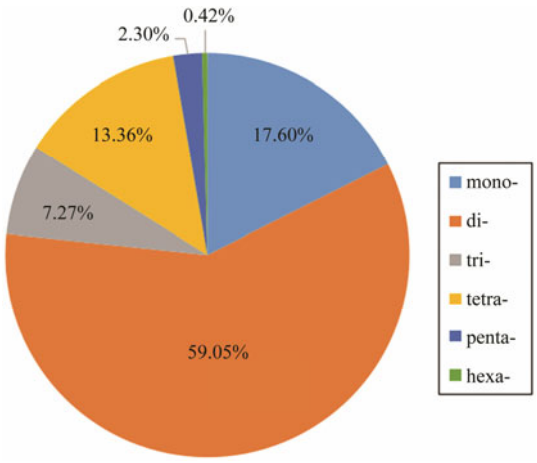

Fig.1 Percentage of different microsatellite repeats in *C. nippona*.

Table 1 Characteristics of 46 microsatellite markers developed for Iwagaki oyster *Crassostrea nippona*

Loci	Repeat motif	Primer sequence (5'-3')	Ta (℃)	Size range (bp)	Na	*Ho*	*He*	*PIC*	P_{HWE}	GenBank accession number
Cn2	(TC)13	F: CCAACCATGATCATCTGGAA R: CTGCATTCTGTCTGGTTTGAA	60	197–229	13	0.633	0.894	0.868	0.0000*	MK078311
Cn7	(GA)13	F: TGCATGGTAACACAATTCCC R: CTTGGAAGGGAATGACTCCA	57	222–276	22	1.000	0.932	0.910	0.0635	MK078312
Cn8	(GA)14	F: AAAAATCTTTTCGCACACGG R: TGCATGGAGGGTTGATCTATT	54	220–240	9	0.567	0.787	0.750	0.0048	MK078313
Cn12	(CA)6	F: CATAGGTGTTGGAACTGGGG R: GGAGCCAAAACATGGTCAAT	62	250–288	13	0.200	0.848	0.817	0.0000*	MK078314
Cn14	(TC)13	F: CCGAGTCGCCAAACAACTAT R: TTGTGGTTTTCTCCGCTCTT	57	205–263	16	0.933	0.928	0.906	0.0651	MK078315
Cn15	(TC)13	F: TCGCTGGTGTTTCTTCATTCT R: CGGAAACCCCTATAGCCAGT	57	223–275	15	0.633	0.898	0.873	0.0000*	MK078316
Cn23	(CT)13	F: CGACAGCAAAGCCACAAATA R: TCGCATAGGCTCCGTAACAT	55	212–244	13	0.733	0.873	0.845	0.0077	MK078317
Cn24	(GAT)9	F: GCCCCTCTGTGGTAAACAAA R: TGTTAAGGATATGCGGGACAA	58	170–200	7	0.467	0.600	0.567	0.0128	MK078318
Cn33	(AG)13	F: GCTCTGATCTCCGCTCTTGT R: TTGTTATTGCGAGCTCCCTT	62	217–275	17	0.933	0.926	0.904	0.7262	MK078319
Cn35	(CT)13	F: CCCACGAATTAACACCCAAG R: TGGGCCTCATGATCTTTTTC	62	311–345	17	0.967	0.902	0.879	0.8272	MK078320
Cn37	(AG)12	F: GGCCTACCTTAATTCAAGCAAC R: AGGGTTAGCAGAGCACAGGA	62	274–350	17	0.400	0.918	0.895	0.0000*	MK078321
Cn38	(TA)8	F: TCGCTCAAAAGGACAACAGA R: TGACCTTCTCCAGCGAGATT	62	191–215	11	1.000	0.877	0.847	0.0007*	MK078322
Cn40	(AG)17	F: CAGAGGAGCAGGAGAAAACG R: GACGTGCGATTTTGTGTCAG	62	208–258	16	0.800	0.925	0.903	0.0000*	MK078323
Cn42	(AG)13	F: GTGATGCTTTATCCTGCCGT R: CTTATGGCGCTGTTTTGGTT	60	309–331	18	0.900	0.928	0.906	0.6523	MK078324
Cn50	(AG)13	F: GGATGAAGCTGGTGATCGTT R: AAACGAACCAAGTTGTGCTG	62	202–238	16	0.967	0.897	0.872	0.0346	MK078325
Cn51	(GAG)8	F: AATCCCTCTCAGGCAGACCT R: TGGAGCTGGACTGGTTCTCT	64	214–232	7	0.667	0.813	0.772	0.1554	MK078326
Cn52	(AG)16	F: TTTCCAAGAGAGAACAACAGAGC R: AGCAATGCATCTCCTTCGAT	64	262–330	19	0.433	0.943	0.923	0.0000*	MK078327

(to be continued)

(continued)

Loci	Repeat motif	Primer sequence (5'–3')	Ta (℃)	Size range (bp)	Na	*Ho*	*He*	*PIC*	P_{HWE}	GenBank accession number
Cn53	(TTA)8	F: TTGCTGCAATTTGTTTGACG R: ATGCACAAAGGCTTTCATCC	64	291–315	13	0.900	0.911	0.887	0.0263	MK078328
Cn57	(AG)17	F: CAGGTGCTGCTTATTATGGAGA R: GCTTCCAGATGCTGCAAACT	64	199–231	15	0.767	0.925	0.903	0.0000*	MK078329
Cn61	(AG)26	F: CAGTTTGGGGGAGAAATTCA R: CAGAAACACGAAAGTTCCGA	62	232–290	16	0.500	0.910	0.886	0.0000*	MK078330
Cn65	(TC)18	F: ATGGGCGACAATAAAACAGG R: TATTGCAACAGCCGTGAGTC	60	269–321	20	0.667	0.920	0.898	0.0000*	MK078331
Cn66	(CT)26	F: ACCAAGACTCGGTTTTGTGC R: GAAACCGATTCGTTAGCACC	58	247–291	12	0.767	0.871	0.840	0.0000*	MK078332
Cn68	(GA)25	F: TTATCGGGAAAATGCTTTCG R: GTCTGTGCGTCTGGAGATCA	62	207–247	13	0.800	0.917	0.893	0.0000*	MK078333
Cn72	(AT)8	F: TCAACTGGTCATTGCTGGTG R: TGCCTGCATGCGTATGTTAT	62	208–240	6	0.500	0.524	0.478	0.4734	MK078334
Cn77	(GTCC)4	F: ATTTGACGGCAGCAGAAATC R: CCAAGAGTTCAGTTTGCTGG	56	217–237	5	0.300	0.693	0.644	0.0000*	MK078335
Cn79	(AG)15	F: CACGTGGAAGTGTGGAAGTG R: AATGGCGGCCTATTGAAAC	62	274–310	12	0.700	0.878	0.850	0.0004*	MK078336
Cn88	(CT)7	F: TTGGACAACAACCTGGGAAT R: ATGTAACATGGCGGGAAAAG	66	189–199	6	1.000	0.765	0.716	0.0016	MK078337
Cn90	(AG)13	F: GCAAGTGAGCATGATTGTCC R: GGGCAGGTTTTTCTAATTTCG	64	247–289	16	0.567	0.908	0.885	0.0000*	MK078338
Cn94	(AG)28	F: TCATTGGATTCCTTGAACACC R: AAACCTCCGGACGAAGAAGT	64	163–203	15	0.667	0.924	0.901	0.0000*	MK078339
Cn96	(AAT)5	F: CATCGCTACATACCCCCTGT R: AACAGACACAGGGAAACGCT	66	251–284	3	0.133	0.525	0.399	0.0000*	MK078340
Cn98	(AG)13	F: TGGATGACGAGCTAATGCAG R: TTCAGCTTGTGCCTCTGTTG	66	278–312	21	0.933	0.949	0.929	0.0000*	MK078341
Cn101	(TAT)7	F: ATCGCAATGTATCTGCGTGA R: AAAAATGACCGTTTTGCCTG	64	268–277	6	0.333	0.508	0.469	0.0048	MK078342
Cn102	(TGA)9	F: ACTGGCATTCGTCGATAACC R: GTCTGCCATCTGCATCGTAA	66	224–236	7	0.700	0.793	0.747	0.0815	MK078343
Cn104	(TGTC)6	F: GTCACATGTTGTCCGTCGTC R: TTCAAGGATGCTGTGTTCCA	66	140–158	6	0.700	0.764	0.712	0.0594	MK078344
Cn105	(AG)11	F: AAGTGGCGGATCATATTGCT R: TAGTGCTTGACGCCATCTTG	64	137–171	8	0.600	0.746	0.701	0.0080	MK078345
Cn107	(TTC)7	F: TCACATTGCCTGTGGTGATT R: ATGACCTTGAAAGACGACCC	60	202–256	12	1.000	0.882	0.853	0.0000*	MK078346
Cn108	(AC)13	F: AATTCAATGTTCGTCGGGAG R: GCTATTGAAGTTTTCCCGCA	64	250–282	9	0.500	0.593	0.555	0.0243	MK078347
Cn112	(AT)8	F: CCTCCGCTTCTTAGACGTTG R: CTGGAGGGAATGTGTGTCCT	64	242–258	6	0.500	0.788	0.738	0.0000*	MK078348
Cn113	(AG)7	F: TGGAGGCATTTCTTGAGTCC R: GAGAGGCATTCGCAAAAGAG	66	176–232	16	0.933	0.899	0.874	0.5445	MK078349
Cn116	(AACAT)4	F: CGGCACTAAGAAACAAAGCA R: GTTTTCAATGCACATCACGG	66	225–235	3	0.567	0.608	0.517	0.1420	MK078350
Cn118	(AG)21	F: CGGATCAAATCCTTCAAAGC R: AATTGCAGAAGTTCCCGATG	66	241–265	12	0.667	0.895	0.869	0.0027	MK078351
Cn133	(CAT)8	F: TCACAACAAAAGGGTGCGTA R: AAAGCCTTGAAAAGTTCCCA	64	124–133	5	0.900	0.738	0.677	0.0298	MK078352
Cn145	(CT)13	F: TTCCATTGTGCATTCTGCAT R: CTGACGTTTCCCCTTCACTC	60	200–262	15	0.900	0.902	0.877	0.5315	MK078353
Cn150	(CT)20	F: TTGTCTCAGCATTCCATTCA R: TGGCCCGTAAATGAACTCTC	60	231–275	17	0.733	0.888	0.862	0.2722	MK078354
Cn151	(TCT)10	F: ATCGATGTGTGGATCCTTCC R: GGCGTCGTAAACATGGATTT	63	270–284	5	0.567	0.455	0.408	0.6479	MK078355
Cn160	(CT)6	F: AATGTCGCAAATTCCAAAGG R: GGCTCAGGAACAATGTGGAT	63	157–173	6	0.633	0.532	0.476	0.6221	MK078356

Notes: Ta, annealing temperature; Na, number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *PIC*, polymorphic information content. * significantly deviated from Hardy-Weinberg equilibrium after sequential Bonferroni's correction ($P < 0.05/46$).

3.2 Multiplex PCR and Parentage Assignments

Fifteen microsatellites were selected and allocated into five optimized multiplex PCR sets that work well for *C. nippona*, each of which contains three microsatellite markers (Table 2). Capillary electrophoresis showed that alleles in each multiplex set could be clearly identified (Fig.2). The polymorphism information and non-exclusion probability of each microsatellite locus were shown in Table 3. Overall, the polymorphism level of the microsatellite loci was moderate, with a mean of 5.7 alleles per locus and a mean *PIC* of 0.575. The *Ho* and *He* varied from 0.169 to

0.814 and 0.236 to 0.804, respectively.

The simulation results using CERVUS 3.0 showed that with these five multiplexes, the total assignment success rate of 97% could be achieved even if the number of candidate parents increased to 300 (Fig.3). In actual parentage assignments, the parentage analysis performed with a cultured population (173 parents and 486 offspring) demonstrated that 80% of all offspring were correctly assigned to their parental pair using three multiplex PCRs with the highest polymorphic information, and 96% of all offspring were allocated to their most likely parents based on all five sets (Fig.4), which was similar to the simulation results. Among 486 offspring, 21 individuals (4%) failed to be assigned to their parents because more than 20% of the loci were not amplified. The most likely reason was that the DNA quanlity was poor.

Table 2 Reaction conditions of five multiplex PCRs

Multiplex sets	Locus	Ta (℃)	Concentration of forward primer ($\mu mol\,L^{-1}$)	Concentration of reverse primer ($\mu mol\,L^{-1}$)
Multiplex set 1	Cn72	60	0.06	0.15
	Cn108	60	0.08	0.20
	Cn42	60	0.06	0.15
	VIC-M13(-21)	53	–	0.20
Multiplex set 2	Cn116	60	0.06	0.15
	Cn101	60	0.06	0.15
	Cn53	60	0.08	0.20
	VIC-M13(-21)	53	–	0.20
Multiplex set 3	Cn133	60	0.06	0.15
	Cn96	60	0.08	0.20
	Cn35	60	0.06	0.15
	FAM-M13(-21)	53	–	0.20
Multiplex set 4	Cn104	60	0.06	0.15
	Cn102	60	0.06	0.15
	Cn98	60	0.08	0.20
	FAM-M13(-21)	53	–	0.20
Multiplex set 5	Cn160	60	0.06	0.15
	Cn118	60	0.06	0.15
	Cn151	60	0.06	0.15
	VIC-M13(-21)	53	–	0.15

Note: Ta, annealing temperature.

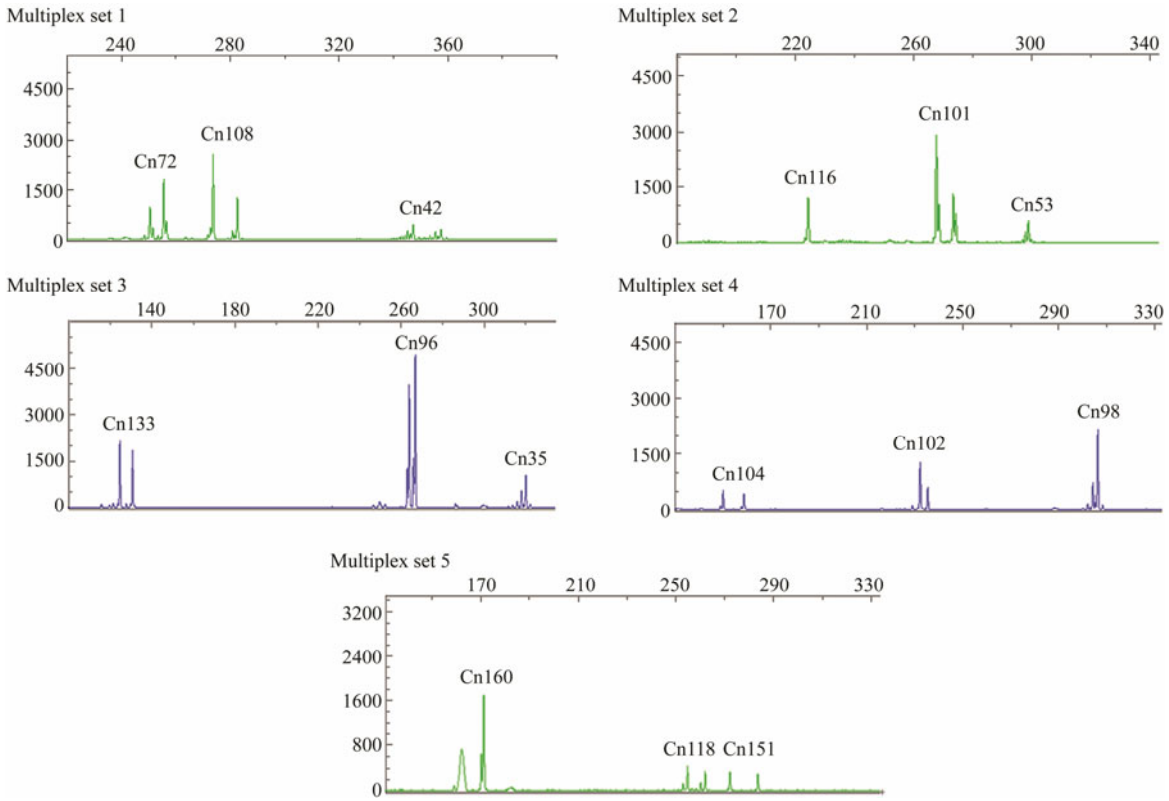

Fig.2 Capillary electrophoresis of five multiplex sets. Horizontal axis shows the size range of each locus in base pairs (bp).

Table 3 Characteristics of the five multiplex PCRs in *Crassostrea nippona*

Multiplex set	Locus	Na	*Ho*	*He*	*PIC*	NE-1P	NE-2P	NE-PP	*F* (Null)
Multiplex set 1	Cn72	4	0.477	0.511	0.397	0.869	0.792	0.686	0.0359
	Cn108	4	0.384	0.429	0.389	0.908	0.774	0.635	0.0572
	Cn42	6	0.814	0.804	0.775	0.566	0.389	0.208	−0.0106
Multiplex set 2	Cn116	3	0.574	0.539	0.481	0.855	0.712	0.562	−0.0439
	Cn101	4	0.592	0.674	0.617	0.750	0.586	0.414	0.0663
	Cn53	6	0.299	0.632	0.558	0.793	0.649	0.493	0.3552
Multiplex set 3	Cn133	4	0.687	0.699	0.645	0.727	0.558	0.383	0.0088
	Cn96	7	0.169	0.528	0.416	0.860	0.776	0.663	0.5188
	Cn35	10	0.554	0.793	0.766	0.571	0.393	0.205	0.1821
Multiplex set 4	Cn104	4	0.751	0.736	0.687	0.688	0.513	0.339	−0.0132
	Cn102	5	0.672	0.737	0.688	0.686	0.512	0.338	0.0470
	Cn98	10	0.595	0.780	0.748	0.599	0.420	0.233	0.1350
Multiplex set 5	Cn160	7	0.721	0.594	0.549	0.806	0.639	0.459	−0.1223
	Cn118	9	0.532	0.743	0.704	0.656	0.478	0.289	0.1577
	Cn151	2	0.267	0.236	0.208	0.972	0.896	0.824	−0.0614

Notes: Na, number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *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; *F* (Null), frequency of null allele.

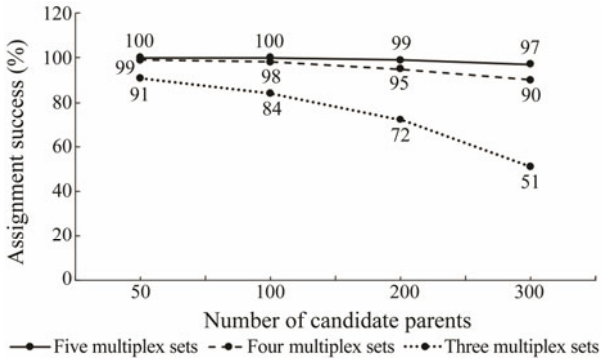

Fig.3 Assignment success rate of simulated genotype data at the 95% confidence level. Each multiplex was added in decreasing order of average polymorphic information content (*PIC*).

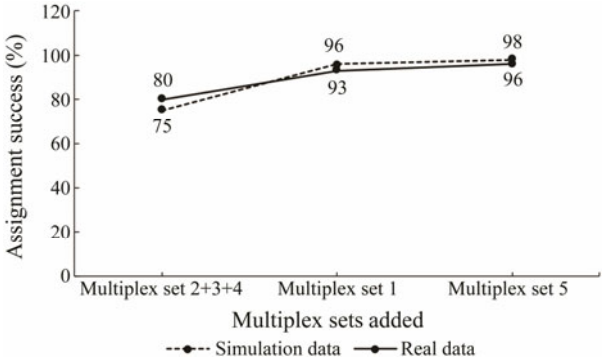

Fig.4 Cumulative assignment success rates of simulated and real genotype data at the 95% confidence level. Each multiplex was added in decreasing order of average polymorphic information content (*PIC*).

3.3 Effective Breeding Number (N_e) Estimation

The results of sibship reconstruction showed that the inferred number of male and female parents were 70 and 63, respectively. We reconstructed full-sib families with COLONY 2.0, indicating that the number of full-sib families were 122 and the number of offspring per family were between 1 to 20. The effective population size (N_e) was estimated as 80.

4 Discussion

Microsatellites are one of the most widely used DNA markers in aquaculture genetics (Liu and Cordes, 2004). The traditional methods of developing genomic microsatellite markers, such as the hybrid capture method (Ostrander *et al*., 1992) and the use of published data (Li *et al*., 2009), are either time-consuming or highly dependent on available resources. With the rapid development on next-generation sequencing technologies, the identification and characterization of large numbers of microsatellites in non-model species have become easier and faster. Next-generation sequencing technique provides a more convenient and economical method for obtaining genetic markers. In this study, a set of 46 polymorphic microsatellite markers were developed and characterized based on RAD-seq. According to the criterion defined by Botstein *et al*. (1980), there are 41 loci with high information content ($PIC>0.5$), and the remaining 5 loci have moderate information content ($0.5<PIC<0.25$). Among 46 loci developed here, 20 loci were significantly deviated from HWE after sequential Bonferroni correction. Many factors can cause the problem, including lack of random mating, sampling effect, and null alleles (Sekino *et al*., 2003).

In order to facilitate large-scale population research, five multiplex PCRs were developed and optimized in the study based on a three-primer-PCR approach, involving a fluorescently labelled universal primer, a specific forward primer with 5'universal primer sequence tails, and a normal specific reverse primer (Blacket *et al*., 2012). Such method not only obtains a level of multiplexing and PCR efficiency similar to microsatellite fluorescent detection assays using direct labeling primers, but also dramatically

reduces the cost of the experiment (Blacket *et al*., 2012). Multiple PCRs developed in this study show relatively high efficiency of parentage assignment. Pedigree analysis of real offspring showed that 80% of the offspring were explicitly assigned to their parents when using three multiplex PCRs. This result is slightly different from previous studies on marine bivalves (Li *et al*., 2010; Nie *et al*., 2012), which found that fewer multiplexes were needed to achieve the same assignment success rate. There are many reasons for this difference, such as the slightly lower *PIC*, the more potential parents and offspring, and unknown parents genders.

The sibship reconstruction revealed that not all candidate parents were involved in the reproduction and the parents participating in reproduction had unequal reproductive contribution. The difference of parental reproductive contribution is the result of various factors, including fertility rate and survival rate of offspring. In this study, the effective size of a population N_e was smaller than the actual number of individuals. Boudry *et al*. (2002) believed that this phenomenon was caused by many factors such as overlapping generations, non-random mating and unbalanced sex ratio. The effective population size is a very important parameter for breeding populations, and its reduction will result in increased inbreeding risk and decrease of genetic diversity of offspring. Thus, in order to reduce the risks of inbreeding and avoid genetic degeneration of hatchery-produced seed, the effective population size per generation in a hatchery population should be increased by increasing the number of parents and equalizing the sex ratio.

In summary, for the first time, we developed and characterizedthe novel polymorphic microsatellites for *C. nippona*. Additionally, five multiplex PCR assays were developed and validated from these new microsatellites using universal primers labeled with fluorescent dyes. As the aquaculture of *C. nippona* is still in its infancy, this study will help to formulate effective management strategies for artificial seeding and breeding. Moreover, the five sets of multiplex PCRs described here can not only be applied to parentage assignment, but also provide an important tool for marker-assisted breeding and population genetic analysis in *C. nippona*.

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