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Contributed Paper

Development of Polymorphic Microsatellites in White Scar Oyster *Crassostrea belcheri*

Nirandon Phuwan [a], Parichart Ninwichian* [a], Srijanya Khemklad [b] and Bavornlak Khamnamtong [c,d]

[a] Faculty of Science and Industrial Technology, Prince of Songkla University, Surat Thani Campus, Surat Thani 84000, Thailand.

[b] Aquatic Animal Genetics Research and Development Division, Khlong 5, Khlong Luang, Pathum Thani 12120, Thailand.

[c] Marine Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand.

[d] Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

* Author for correspondence; e-mail: parichartn@yahoo.co.uk

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ABSTRACT

The white scar oyster *Crassostrea belcheri* is one of economically important species in Thailand. To improve the management efficiency of *C. belcheri*, appropriate molecular markers are needed to be developed. In this study, microsatellites in *C. belcheri* were cross-amplified using type I microsatellites designed from express sequence tag (EST) of *Crassostrea gigas*. In total, 60 EST-derived microsatellites of *C. gigas* were tested and 41 loci (68.33%) generated the amplification product against genomic DNA of *C. belcheri*. Of these, 18 loci (43.90%) were polymorphic and were preliminary screened for estimation of a polymorphic level against *C. belcheri* originating from Surat Thani (N = 50). The number of alleles per locus ranged from 4 to 15 with an average number of 8.83 alleles per locus. The observed heterozygosity varied from 0.0000 to 0.8571 with an average of 0.3982 whereas the expected heterozygosity ranged from 0.4149 to 0.8930 with an average of 0.7487. These polymorphic microsatellites were further tested against bulked genomic DNA (5 bulks with 10 individuals for each bulk) from 5 different geographic locations. Thirteen pairs of primers generated the positive amplification products across all examined geographic samples while two pairs of primers yielded the amplification products only in the Surat Thani sample and they were regarded as candidate population-specific markers. Microsatellites in the present study provide a valuable fundamental resource to facilitate further research on population genetics and stock management of *C. belcheri* in Thailand.

Keywords: cross-species amplification, genetic variation, microsatellite, oyster

1. INTRODUCTION

The white scar oyster (*Crassostrea belcheri*) is economically important species in Thailand and widely cultured in parts of near shores, shallow-water bays, and estuaries. To improve the efficiency of *C. belcheri* production, molecular tools are needed for sustainable farm management. Species-specific markers for differentiation of commercially cultured oysters (*C. belcheri*, *Crassostrea iredalei* and *Saccostrea cucullata*) in Thailand have been developed based on randomly amplified polymorphic DNA (RAPD) [1] and restriction fragment length polymorphism (RFLP) of mitochondrial DNA genes [2]. Nevertheless, intraspecific genetic diversity which is the fundamental information for broodstock management, conservation, and genetic improvement program of *C. belcheri* was only studied by allozyme analysis [3].

Microsatellites are powerful for population genetic studies in various taxa [4]. Microsatellites are short tandemly repeated (1-6 nucleotides) DNAs, abundantly dispersed in eukaryotic genomes [5]. Unlike mitochondrial DNA which are transmitted maternally, RAPD and AFLP which are treated as dominant markers, microsatellites segregate in a co-dominant fashion allowing the differentiation between homo- and heterozgotes at each locus. The levels of microsatellite polymorphism vary greatly among loci but are often higher than those observed in allozymes and mtDNA [6]. Highly polymorphic loci exhibiting large numbers of alleles are ideal for gene mapping and pedigree analysis [7-8], whereas loci with lower levels of polymorphism can be used for analysis of population differentiation [9]. However, no information of genetic diversity in *C. belcheri* based on microsatellites is available at present and prior to this study no microsatellites have been developed and studied in *C. belcheri*. Thus, there is a need to develop microsatellites to study genetic diversity in *C. belcheri*.

The development of microsatellite markers for a new species using traditional methods is tedious and time consuming [10]. With the abundance and availability of genetic information presented in the public database, expressed sequence tags (ESTs) become the useful resource for identifying type I (coding) microsatellites especially in non-model species [11]. Microsatellite screening from EST database of the closely related species and/or the previously published microsatellite markers become more common and widely used approach [12]. The advantages of EST-derived microsatellites are that they allow higher cross-species amplification, exhibit lower frequency of null alleles, and provide less multiple band problem than genomic-derived type II microsatellites [13]. In addition, polymorphism of coding microsatellites may associate with interested phenotypes allowing the application using in selective breeding programs of species under study.

Identifying informative markers through cross-species amplification provided an alternative way to overcome the paucity of genetic information in non-model species. Previously, cross-species amplification using EST-derived microsatellites has been reported in some oyster species such as *Crassostrea gigas*, *Crassostrea rhizophorae*, *Crassostrea ariakensis*, and *Crassostrea sikamea* [14]. The success in identifying informative microsatellites using cross-species amplification in non-model species provides a valuable resource for genetic variability, conservation, population genetic studies, parentage analysis, genome and comparative mapping. In this study, we evaluated the cross-species amplification

of microsatellites derived from ESTs of *C. gigas* in *C. belcheri*. Polymorphism of various microsatellites was examined in a population from Surat Thani, the main production source of oyster larvae in Thailand. In addition, bulk segregant analysis (BSA) of genomic DNA from 5 different geographic locations were screened for further application on population genetic studies of this economically important species in Thailand.

2. MATERIALS AND METHODS

2.1 Sample Collection

Wild *C. belcheri* was collected from Bandon Bay, Kanchanadit, Surat Thani province (N = 50) and Lam Phaen canal, Laem Sing, Chanthaburi province (N = 10) located in the Gulf of Thailand and from Palian river, Kantang, Trang province (N = 10), Now canal, Khura Buri, Phang Nga province (N = 10), and Bang Man canal, Suk Samran, Ranong province (N = 10) located in the Andaman Sea (Figure 1). Mantle tissues of each individual were dissected out and cut to small pieces (2×2 mm), preserved in absolute ethanol and stored at -20 °C until further need. This study was approved by the Animal Ethics Committee of Prince of Songkla University and the experiment procedures were performed according to the National Research Council of Thailand guidelines for the use of laboratory animals.

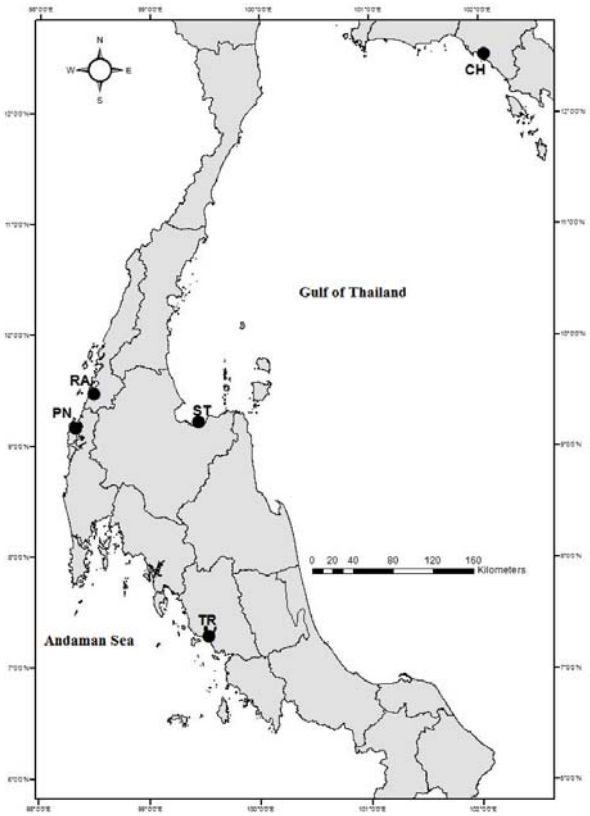

Figure 1. A map showing sampling sites of *Crassostrea belcheri* in Thai waters in this study. Two geographic samples were from the Gulf of Thailand (ST, Surat Thani and CH, Chanthaburi) and three samples were from the Andaman Sea (TR, Trang; PN, Phang Nga; RA, Ranong). The sampling sites were indicated in the solid dots.

2.2 DNA Extraction

A piece of mantle tissue was transferred into a 1.5 mL Eppendorf tube containing 600 µl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 1.2M NaCl; pH 8.0, 0.2% TritonX 100 and 200 µg/ml proteinase K). Tissue was homogenized using a micropestle and placed in 55 °C in a water bath for 3 h or until all tissues were completely dissolved. Genomic DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with an equal volume of chloroform-isoamyl alcohol (24:1). DNA was subsequently precipitated with two volumes of cold absolute ethanol. DNA pellets were washed in cold 70% ethanol, air dried, and resuspended in 30 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The DNA quality of extracted DNA was visualized by 1% agarose gel electrophoresis. The purity and quantity of extracted DNA were spectrophotometrically measured. DNA was kept at -20 °C until analysis.

2.3 Sources of Microsatellite Markers and Primer Design

A total of 60 EST microsatellite primer pairs were used to examine the cross-species amplification in the present study. Of these, 30 microsatellite primer pairs were selected from previously published EST-derived microsatellites of *C. gigas* based on their high polymorphism. To obtain additional microsatellites, a total of 300 non-redundant EST sequences of *C. gigas* available in the GenBank were downloaded and searched for microsatellites using the Msatcommander software [15]. EST containing microsatellites with at least 50 bp of flanking sequences on either side of microsatellites were selected for primer design. A total of 30 microsatellite primer pairs were designed by Msatcommander with the expected amplification product sizes between 200-350 bp.

2.4 Cross-species Amplification of *C. gigas*-originated Microsatellites in *C. belcheri*

Sixty primer pairs of *C. gigas* were initially used to determine the efficacy of cross-species amplification against 5 individuals of *C. belcheri* from Surat Thani. PCR was performed in a 10 µl reaction mixture containing 5 ng genomic DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 µl of 10 pmol/µl of each primer, and 1 U of *Taq* polymerase (Invitrogen). Two-step PCR profiles were used for amplification with an initial denaturation step at 94 °C for 4.0 min followed by denaturation at 94 °C for 45 s, annealing at 42 °C for 45 s, and extension at 72 °C for 90 s for 10 cycles. The thermal cycling was performed for additional 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s. The final extension step was performed at 72 °C for 10 min. In this two-step PCR profile, the annealing temperature window is accommodated to determine the best T_m for the primer pairs, except locus CgTH28, the annealing temperature was 50 °C for 40 cycles. The presence or absence of DNA fingerprints was initially screened by separated the amplification products on 2% agarose gel electrophoresis at 100 V for 25 min and visualized under the UV light after staining with *SYBR® Safe*. The amplification quality was classified into 3 quality classes as described in Liewlaksaneeyanawin et al. [13]. Amplified microsatellite loci were subsequently analyzed across 50 individuals of *C. belcheri* collected from Surat Thani on a 6% denaturing polyacrylamide gel electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer and visualized by silver

staining. Sizes of the PCR products were compared with pBR322/MspI DNA ladder.

2.5 Preliminary Determination of Polymorphic Microsatellite Loci Through BSA

A total of 5 bulks of *C. belcheri* collected from Surat Thani (ST), Chanthaburi (CH), Trang (TR), Phang Nga (PN), and Ranong (RA) were screened. Each bulk contained an equal quantity of genomic DNA (25 ng/µl) collected from 10 individuals of *C. belcheri*. PCR amplification was subsequently performed in all bulks to preliminary screen for polymorphic microsatellites. PCR products were separated on 2% agarose gels and compared with a 100 bp DNA ladder to estimate allele size ranges. DNA banding patterns of each bulk from each locus were used for evaluating whether it was polymorphic or monomorphic. The PCR amplification efficiency was classified into 4 quality classes of amplification criteria as follows: +++ = very good amplification; ++ = good amplification; + = weak amplification; - = no amplification.

2.6 Data Analysis

Clear amplification alleles of each locus were scored. Genetic parameters including the number of allele per locus (N_a), observed (H_o) and expected (H_e) heterozygosity and effective number of alleles (N_e) were calculated and analyzed using POPGENE version 1.32 [16]. Inter-individual fixation index (F_{is}) [17], the probability tests for the Hardy-Weinberg equilibrium (HWE), and pairwise linkage disequilibrium among loci were calculated and analyzed using Genepop software available from http://genepop.curtin.edu.au/index.html. The presences of null alleles at each locus were determined using MICRO CHECKER 2.2.3 software [18].

3. RESULTS AND DISCUSSION

3.1 Amplification of *C. belcheri* Microsatellites using Heterospecific Primers from *C. gigas*

Although *C. belcheri* is one of economically important oyster species in Thailand, information of its intraspecific genetic diversity based on microsatellite polymorphism is not available. With the availability of EST-derived microsatellites in *C. gigas* and *Crassostrea virginica*, the information could be used as a valuable resource for cross-species amplification in *C. belcheri*. However, phylogenetic relationships based on cytochrome oxidase c subunit I (COI) sequences of *C. gigas* were closer to *C. belcheri* than *C. virginica* [19]. Accordingly, the development of microsatellite primers in this study was performed using those from *C. gigas*.

Cross-species amplification of 60 primer pairs designed from microsatellite-containing ESTs of *C. gigas* against genomic DNA of *C. belcheri* was summarized in Table 1. Among 60 EST microsatellite loci, 19 loci (31.67%) did not generate the amplification product while 41 loci (68.33%) were successfully amplified in *C. belcheri*. Of these, 18 loci (43.90%) were polymorphic and 1 locus (2.44%) was monomorphic, 12 loci (29.27%) generated multiple alleles, and 10 loci (24.39%) produced weak amplification results. Among 18 polymorphic loci established, 10 loci (55.56%) were derived from previously published microsatellite primers while the remaining 8 loci (44.44%) were newly developed from the EST-database.

Table 1. Cross-species amplification in *Crassostrea belcheri* using EST microsatellites of *Crassostrea gigas*.

Loci	Accession no.	Reference	Repeat motif	Quality class
CgF114	AM854045	Bai et al. 2011 [25]	$(TG)_5$	3(N)
CgL12	AM859900	Bai et al. 2011 [25]	$(TA)_7$	3(N)
CgL40	EW777557	Bai et al. 2011 [25]	$(TC)_{13}C(CT)_7$	3(N)
Cgsili4	AM854894	Sauvage et al. 2009 [26]	$(AG)_{26}$	3(N)
Cgsili6	AM854296	Sauvage et al. 2009 [26]	$(GA)_{26}$	3(N)
Cgsili7	CU682571	Sauvage et al. 2009 [26]	$(GA)_{24}$	1
CgE315	AM855806	Yu et al. 2010 [23]	$(TC)_5(CT)_9$	3(N)
CgE317	AM856446	Yu et al. 2010 [23]	$(AG)_6\ldots(AG)_{18}$	3(M)
CgE322	AM854894	Yu et al. 2010 [23]	$(GA)_{26}...(GA)_7$	3(N)
CgE335	AM861511	Yu et al. 2010 [23]	$(GA)_{27}$	3(M)
CGE014	BQ426727	Yu and Li 2008 [27]	$(TG)_5$	3(N)
CGE020	DV736352	Yu and Li 2008 [27]	$(TA)_6$	3(N)
CGE021	DV736668	Yu and Li 2008 [27]	$(TG)_5$	3(N)
CGE024	BQ427306	Yu and Li 2008 [27]	$(AT)_5$	3(N)
CGG008	AJ579915	Li et al. 2009 [12]	$(AG)_{20}$	1
CGE027	ES789161	Li et al. 2009 [12]	$(AG)_5$	1
CGE032	CX068987	Li et al. 2009 [12]	$(AG)_7$	3(M)
CgEX45	FP002613	Li et al. 2011 [28]	$(TC)_5$	1
CgEH05	FP001717	Li et al. 2011 [28]	$(AT)_{10}$	3(W)
CgEH10	FP004026	Li et al. 2011 [28]	$(GA)_{14}$	3(W)
CgEH19	CU991737	Li et al. 2011 [28]	$(GAA)_7$	1
CgEH81	CU993748	Li et al. 2011 [28]	$(AG)_{24}$	1
CgEH32	CU999674	Li et al. 2011 [28]	$(TA)_5$	1
CgEH57	CU996627	Li et al. 2011 [28]	$(AT)_7$	1
CgEH67	CU996272	Li et al. 2011 [28]	$(CAG)_5$	1
CgEH143	CU990160	Li et al. 2011 [28]	$(AG)_{11}$	3(W)
CgEH148	CU986337	Li et al. 2011 [28]	$(GA)_5$	1
CgEH149	CU989133	Li et al. 2011 [28]	$(AT)_7$	3(W)
CgEH154	FP011198	Li et al. 2011 [28]	$(GAA)_6(GAT)_5$	3(N)
CgEH167	CU991657	Li et al. 2011 [28]	$(TA)_5$	2
CgTH01	AM853207	Tanguy et al. 2008 [29]	$(AGAT)_{18}$	3(N)
CgTH02	AM853553	Tanguy et al. 2008 [29]	$(AC)_6$	3(W)
CgTH03	AM857594	Tanguy et al. 2008 [29]	$(GAT)_5$	3(M)
CgTH04	AM860740	Tanguy et al. 2008 [29]	$(TG)_6$	1
CgTH05	AM863488	Tanguy et al. 2008 [29]	$(CT)_5$	3(N)
CgTH06	EY470957	Mitreva et al. 2005 [30]	$(AGG)_6$	1
CgTH07	CU991497	Genoscope 2009 [31]	$(GT)_5$	3(N)
CgTH08	CU992795	Genoscope 2009 [31]	$(GT)_5$	1
CgTH09	CU992874	Genoscope 2009 [31]	$(GT)_5$	3(M)
CgTH10	FP005493	Genoscope 2009 [31]	$(GT)_5$	1

Table 1. Continued.

Loci	Accession no.	Reference	Repeat motif	Quality class
CgTH11	FP005504	Genoscope 2009 [31]	$(AAAAC)_3$	3(M)
CgTH12	HS116142	Lucas et al. 2010 [32]	$(GA)_5$	3(N)
CgTH13	HS116313	Lucas et al. 2010 [32]	$(TA)_7$	3(M)
CgTH14	HS116434	Lucas et al. 2010 [32]	$(AT)_5$	3(W)
CgTH15	HS116663	Lucas et al. 2010 [32]	$(GAA)_4$	1
CgTH16	HS116779	Lucas et al. 2010 [32]	$(AG)_6$	1
CgTH17	HS116899	Lucas et al. 2010 [32]	$(AG)_5$	3(W)
CgTH18	HS116954	Lucas et al. 2010 [32]	$(TA)_5$	3(N)
CgTH19	HS116977	Lucas et al. 2010 [32]	$(TC)_6$	3(W)
CgTH20	HS116988	Lucas et al. 2010 [32]	$(TA)_6$	1
CgTH21	HS117068	Lucas et al. 2010 [32]	$(CCA)_6$	3(N)
CgTH22	HS118802	Lucas et al. 2010 [32]	$(AAG)_5$	3(M)
CgTH23	HS118866	Lucas et al. 2010 [32]	$(TA)_4$	3(W)
CgTH24	HS119451	Lucas et al. 2010 [32]	$(AAC)_5$	3(M)
CgTH25	HS119540	Lucas et al. 2010 [32]	$(AG)_6$	3(M)
CgTH26	HS119590	Lucas et al. 2010 [32]	$(AGC)_5$	3(N)
CgTH27	HS119616	Lucas et al. 2010 [32]	$(AAG)_5$	3(M)
CgTH28	HS119690	Lucas et al. 2010 [32]	$(AAAAC)_3$	1
CgTH29	HS119755	Lucas et al. 2010 [32]	$(AG)_5$	3(W)
CgTH30	HS119761	Lucas et al. 2010 [32]	$(AAG)_5$	3(M)

* CgTH01 - CgTH30 were newly designed from ESTs in the GenBank database. Quality class of microsatellite: 1 = polymorphism; 2 = monomorphism; 3 = poor amplification (M = multiple alleles; N = null alleles; W = weak amplification).

The ability of cross-species amplification using microsatellites among closely related species has been previously reported in several *Crassostrea* species such as *Crassostrea plicatula*, *Crassostrea hongkongensis*, *Crassostrea nippona*, *C. ariakensis* and *C. sikamea* [12]. Considering the use of EST-derived microsatellites of *C. gigas* from cross amplification in other species, the rate of amplification success in *C. belcheri* was lower than that observed in *C. plicatula* (93.33%), *C. sikamea* (93.33%), *C. hongkongensis* (80%), *C. nippona* (80%), and *C. ariakensis* (73.33%) [12]. Melo et al. [19] reported that *C. gigas* was genetically closer to *C. nippona* and *C. ariakensis* than *C. belcheri*. Nevertheless, the cross amplification success in *C. belcheri* using heterospecific primers designed from ESTs of *C. gigas* was still greater than those using primers designed from genomic sequences (e.g 30.77% in *C. gigas* and 38.44% in *C. ariakensis* [20]). A possible explanation for this could be related to the conservation of the transcribed regions in ESTs, leading to higher cross-species amplification than using genomic derived microsatellites.

3.2 Genetic Variability of *C. belcheri* in the Surat Thani Sample

Genetic variation of *C. belcheri* from Surat Thani ($N = 50$) were analyzed using 18 microsatellites (Table 2). All loci generated the PCR products within the expected size ranges as indicated in *C. gigas*. The number of alleles

per locus (N_a) ranged from 4 (CgTH06) to 15 (CgEH148) with an average number of 8.83 alleles per locus. The observed heterozygosity (H_o) varied from 0.0000 to 0.8571 with an average of 0.3982 while the expected heterozygosity (H_e) ranging from 0.4149 to 0.8930 with an average of 0.7487. The effective number of alleles per locus (N_e) varied from 1.6551 to 8.5207 with an average effective number of 4.3575 alleles per locus. Apparently, the effective number of alleles from each locus was lower than the number of alleles, indicating that the presence of rare alleles at each locus.

Table 2. Polymorphism of 18 heterospecific microsatellite loci derived from ESTs of *Crassostrea gigas* and amplified against genomic DNA of *Crassostrea belcheri* originating from Surat Thani.

Loci	Repeat motif	Primer sequence (5'-3')	Allele size range (bp)	N_a	H_o	H_e	N_e	F_{is}
Cgsili7	$(GA)_{24}$	F:ACAACGCTATCAGAACCATT R:ATCTCCCGGCAAGTATATG	340-404	10	0.6400	0.6558	2.8506	0.0243*
CGE027	$(AG)_5$	F:GCCGCCTTTCAGACTTTC R:GATGGGACAAACAACGACA	356-388	10	0.0465	0.8930	8.5207	0.9485*
CGG008	$(AG)_{20}$	F:TCTCCTCTACCCCGACAG R:GTGATGAACAAACCACCAAC	304-344	5	0.2051	0.6620	2.8861	0.6929*
CgEX45	$(TC)_5$	F:GAGTCCATCTGCTTCAACA R:GAGTCCATCTGCTTCAACA	334-362	9	0.2222	0.7792	4.2507	0.7187*
CgEH19	$(GAA)_7$	F:CCTTCATTGTTGCTGCTATTGTT R:CTGTCAATTTCCCAGCTACA	261-348	10	0.6122	0.6617	2.8980	0.0754*
CgEH32	$(TA)_5$	F:TTACTTGCCGCTGACTTTCT R:GGTTGACATGCCGTGTAATC	254-272	10	0.4255	0.8243	5.4209	0.4865*
CgEH57	$(AT)_7$	F:GGTGATATTGACGCTGTGCTC R:ACATGGCATTAGTGGCAGGT	352-480	7	0.1463	0.7974	4.7087	0.8183*
CgEH67	$(CAG)_5$	F:CCGAGTCCCCATCATTACCT R:TGTTTTGGAGTTTGTACCTGTGC	180-339	11	0.8571	0.7843	4.3907	-0.0956*
CgEH81	$(AG)_{24}$	F:CTCGGGAAACTACAGCAGAC R:ATTCATCGTTCACTACAACACTC	236-260	5	0.0000	0.7083	3.3419	1.0000*
CgEH148	$(GA)_5$	F:CTGCCATCATTGCTCCCTAC P:ATGGTGCCCTGATCTGCTT	422-512	15	0.3830	0.8451	6.1022	0.5495*
CgTH04	$(TG)_6$	F:CCTTCCTATCTGGTGCTTTACG R:TGGCTACATTATGCTGATTTCTACAG	280-306	9	0.4634	0.7206	3.4696	0.3597*
CgTH06	$(AGG)_6$	F:CTGATGGTGCGAAACCACG R:GGTGAGGCGACTCGTAGAC	399-441	4	0.0800	0.4149	1.6551	0.8512*
CgTH08	$(GT)_5$	F:CGGGACCAAATGGTTGTGC R:CAATGAGATAAGGGCACTGGG	326-392	7	0.5833	0.7963	4.7165	0.2695*
CgTH10	$(GT)_5$	F:CACACCCTTCCACAAGCTG R:TAGCACCGACATACGGGAC	346-384	13	0.8000	0.7495	3.8760	-0.0681*
CgTH15	$(GAA)_4$	F:CAGGAGCAAGCCAAGGAC R:GGTCGGCTCTGATAATGGC	525-582	7	0.0476	0.7263	3.5422	0.9352*
CgTH16	$(AG)_6$	F:GATCGTTTGGTGACAGGCG R:CCTCCCTGCCAGTTATCCC	261-306	9	0.5957	0.8202	5.3037	0.2758*
CgTH20	$(TA)_6$	F:CCGCTGCCAAAGTGAACAG R:ATCCTGGAGTGCTTGGCTC	210-264	10	0.6200	0.8119	5.0968	0.2382*
CgTH28	$(AAAAC)_3$	F:TCTCAGGCTGTCCTATTATTGAG R:TCTTGGCAGCAAACAAGGTG	246-300	8	0.4390	0.8251	5.4051	0.4710*

N_a= number of alleles; H_o= observed heterozygosity; H_e= expected heterozygosity; F= forward primer; R= reverse primer;
N_e = Effective number of alleles; F_{is} = inter-individual fixation index, * means *p*-value < 0.05 indicating significant departure from Hardy-Weinberg equilibrium.

The inter-individual fixation index (F_{is}) ranged from -0.0956 to 1. Of these, 16 loci provided the positive F_{is} while the remaining 2 loci represented the negative F_{is}. Linkage disequilibrium was detected between CgEH32-CgTH20, CgTH10-CgTH20, and CgTH06-CgTH10 ($P < 0.05$). All polymorphic microsatellite loci displayed significant deviations from Hardy-Weinberg equilibrium (HWE) ($P < 0.05$) where 16 loci were resulted from homozygote excess and 2 loci (CgEH67 and CgTH10) were from heterozygote excess. Microchecker analysis revealed that the evidence of null alleles caused homozygote excess at these loci. Similar circumstance of Hardy-Weinberg disequilibrium has been observed in a number of marine organisms such as *C. virginica* [21], *C. gigas* [12], and *Holothuria scabra* [22].

The polymorphic level of type I microsatellites in the Surat Thani sample was higher than those in *C. gigas* (mean N_a = 5.59 per locus; mean H_e = 0.6812) [23], *C. hongkongensis* (mean N_a = 6.62 per locus; mean H_e = 0.7848) [20], and *Meretrix petechialis* (mean N_a = 6.40 per locus; mean H_e = 0.1881) [24], suggesting that high levels of genetic variation should be found in Thai *C. belcheri*. In addition, mean N_a (8.83) in this geographic sample was higher than that of *C. belcheri* in Thai waters (N_a = 2.33) inferred from allozymes previously reported in Day et al. [3].

3.3 Application of BSA for Rapid Evaluation of Polymorphic Microsatellites Across *C. belcheri* from Different Geographical Origins

Eighteen polymorphic microsatellite loci were further tested for preliminary screening whether they could be used for population genetic studies of *C. belcheri* in Thai waters. For rapid screening, BSA was performed using pooled genomic DNA of oysters from each sample (N = 10 for each). The amplification efficiency and size-range of the amplification products was illustrated in Table 3 and Figure 2 as an example. Eight loci (Cgsili7, CgEH19, CgEH32, CgEH57, CgTH04, CgTH06, CgTH10, and CgTH20) provided good amplification efficiency with the intense amplification product across all samples. In addition, 5 loci (CGE027, CgEX45, CgEH81, CgEH148, and CgTH15) produced relatively weak amplification in at least one bulked DNA. Regarding the electrophoresed band patterns, 12 loci were polymorphic and only 1 locus was monomorphic. The remaining loci (CGG008, CgEH67, CgTH08, CgTH16, and CgTH28) failed to generate the amplification products in at least one bulked DNA. Apparently, loci CGG008 and CgTH08 were amplified only in the Surat Thani bulked DNA, suggesting that these loci might be specific to *C. belcheri* collected from Surat Thani. Furthermore, these loci might have the potential to use as the informative markers to discriminate *C. belcheri* of Surat Thani population from the other populations. However, this should be further tested against large sample sizes of *C. belcheri* originating from different locations in Thailand at the individual level. Overall, all informative microsatellite markers preliminary screened through BSA would be useful for further genetic studies in *C. belcheri*.

Table 3. The amplification efficiency and estimated size range (bp) of the amplification products tested across 5 bulked DNA of *Crassostrea belcheri* from 5 different geographic locations.

Locus	Geographic sample						Size range (bp)					
	Surat	Thani	Chanthaburi	Trang	Phang Nga	Ranong	Surat	Thani	Chanthaburi	Trang	Phang Nga	Ranong
Cgsil7	+++		+++	+++	+++	+++	350-380		360-380	360-400	350-370	350-380
CGE027	+++		+	+++	+++	+++	350-380		350-380	300-350	370-380	360-380
CGG008	++		-	-	-	-	320-330		-	-	-	-
CgEX45	++		++	++	+	++	310-320		310-320	300-320	320-330	300-320
CgEH19	+++		+++	+++	+++	+++	310-330		320-340	315-350	320-350	320-330
CgEH32	+++		+++	++	+++	+++	230-250		240-250	250-310	270-280	250-280
CgEH57	+++		+++	+++	+++	+++	350-360		350-360	350-360	350-360	350-360
CgEH67	+++		-	+++	+	+++	180-190		-	180/200	180-200	190-200
CgEH81	++		+	+	+	+	230-240		230-250	270-290	200-210	200-210
CgEH148	+++		+	+++	+++	+++	420-450		420-450	420-450	430-440	410-450
CgTH04	+++		+++	+++	+++	+++	280-290		290-300	250-260	270-290	290-300
CgTH06	+++		+++	+++	+++	+++	400-410		370-390	370-390	380-400	390-400
CgTH08	++		-	-	-	-	320-330		-	-	-	-
CgTH10	+++		++	+++	+++	+++	350-370		330-350	560-600	350-370	360-370
CgTH15	++		+	+	+	+	520-540		500-520	500-550	520-540	520-540
CgTH16	+++		-	-	++	-	260-280		-	-	250-260	-
CgTH20	+++		+++	+++	+++	+++	220-240		250-260	230-240	220-250	220-230
CgTH28	++		-	-	++	++	240-250		-	-	240-250	250-260

Amplification efficiency: +++ = very good amplification; ++ = good amplification; + = weak amplification; - = no amplification

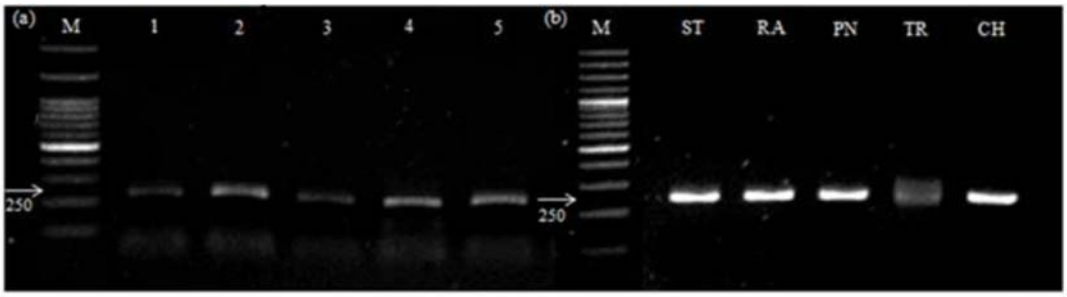

Figure 2. The amplification results of CgEH32 against bulked genomic DNA of *Crassostrea belcheri* from different geographic locations. The PCR products were separated on 2% agarose gel run in 1x TBE buffer at a constant voltage of 100 V for 25 min. Lanes 1-5 = genomic DNA of 5 individuals of *C. belcheri* from Surat Thani (a). Lanes ST, RA, PN, TR, and CH = pooled genomic DNA (*N* = 10 per bulk) from Surat Thani, Ranong, Phang Nga; Trang, and Chanthaburi, respectively (b). Lanes M = a 100 bp DNA ladder.

4. CONCLUSION

Cross-species amplification using EST microsatellite markers of *C. gigas* revealed the cross-species amplification success in *C. belcheri*, providing time and cost savings for polymorphic marker development. Eighteen polymorphic microsatellite loci identified in this study revealed the relatively high genetic variation within *C. belcheri* population collected from Surat Thani, suggesting that high levels of genetic variation should exist in the wild *C. belcheri* population from Surat Thani. Finally, BSA is a simple and rapid approach for detection of polymorphic microsatellite loci across *C. belcheri* from five examined geographic regions. Consequently, polymorphic microsatellite loci obtained in this study will provide valuable information for population genetic study, stock management, and other relevant genetic studies of *C. belcheri*.

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