Aquaculture
December 2010, Volume 310, Issues 1-2, Pages 234-239
http://dx.doi.org/10.1016/j.aquaculture.2010.09.037

Development of four EST-SSR multiplex PCRs in the Pacific oyster (*Crassostrea gigas*) and their validation in parentage assignment

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Abstract:

We report four highly informative multiplex PCRs developed from 12 previously described EST-SSRs in *Crassostrea gigas*. We evaluated and validated these multiplex PCRs in 12 full-sib families. The average allelic richness and the polymorphism information content (PIC) were 11.1 and 0.811 respectively. The combined power of exclusion was greater than 99.99% using all four multiplex assays. A hundred and forty three tests of segregation ratios revealed 11 significant departures from expected Mendelian ratios. The frequency of null alleles was estimated as 4.9% of all the alleles segregating based on a within-family analysis of Mendelian segregation patterns. Parentage analysis of real offspring demonstrated that 97% of all offspring were unambiguously allocated to a pair of parents based on two multiplex PCRs with only a 4% error rate, and 100% of the offspring were correctly allocated to their parents when three multiplex PCRs were used.

Keywords: Pacific oyster; *Crassostrea gigas*; Microsatellite; EST; Multiplex PCR; Parentage assignment

1. Introduction

The Pacific oyster, *Crassostrea gigas*, is the main cultivated oyster species worldwide with a global production of 4.2 million metric tons in 2007 (FAO, 2010). A great deal of genetic research has been conducted to improve the production of this species (Camara et al., 2008; Dégremont et al., 2007; Desrosier et al., 1993; Evans and Langdon, 2006; Guo et al., 1994; Hubert and Hedgecock, 2004; Langdon et al., 2003; Li et al., 2006; Sauvage et al., 2010; Tanguy et al., 2008). In particular, significant mortality of the *C. gigas* has been reported during the summer months in Japan, USA and France. (Mori, 1979; Perdue and Erickson, 1984; Samain and McCombie, 2008), and survival of juvenile Pacific oysters under summer mortality conditions has a strong genetic basis (Dégremont et al., 2007), which was successfully confirmed over several generations through a divergent selection (Dégremont et al., 2010). The genetic improvement based on parentage selection and marker assisted selection was considered to be one of the methods to reduce the mortality of this species and consequently increase the production.

Simple sequence repeats (SSRs; also called microsatellites) have been growing in popularity in genetic analysis because of their high level of polymorphism and codominant characterization. The effectiveness of the SSRs in parentage analysis of *C. gigas* has been proved (Boudry et al., 2002; Li R et al., 2009; Matson et al., 2008). Simple sequence repeats derived from expressed sequence tag (EST) databases (EST-SSRs) have a number of advantages, such as higher transferability across a broader taxonomic range, lower frequencies of null alleles, and association with known function genes (Liu et al., 1999; Pashley et al., 2006). Thus they could be valuable for studies of local adaptation, population structure, selective breeding programs, parentage analysis and genome mapping.

The multiplex PCR technique not only reduces the time and cost associated with SSRs analysis, but also decreases the repeated manipulation of large numbers of sample during the genotyping and, therefore, the risk of handling errors (Porta et al., 2006). However, in *C. gigas*, only one multiplex developed from genomic DNA was reported (Taris et al., 2005).

In this paper, we first reported the development of multiplex PCRs from previously described EST-SSRs of *C. gigas* and their power in parentage assignment was validated in twelve single-pair mating families.

2. Material and methods

Twelve *C. gigas* single-pair mating families were produced from genitors selected for summer mortality or reproduction behavior in February 2009 in Ifremer La Tremblade, France. They were used to test the resolving power of the four EST-SSR multiplex PCRs. For each family, gill samples of both parents and 23 offspring, randomly sampled at one year old, were stored in 70% ethanol until DNA extraction. Genomic DNA was extracted from gill tissue by the chloroform/isoamylalcohol method and purified with the DNA Clean Up System (Promega, Madison, WI, USA). Quality and concentration of each DNA sample were assessed using a spectrophotometer and by running a small amount on a 1% agarose gel.

Eleven sets of primers were selected from previously described expressed sequence tag–derived SSRs (Li Q et al., 2009; Sauvage et al., 2009; Yu and Li, 2007) according to their polymorphism, linkage information, sequence motif repeats and amplification behavior. A SSR developed from *C. gigas* amylase gene was also incorporated in this study (Sellos et al., 2003). One primer of each pair was labeled with one fluorescent dye (HEX, FAM or NED).

The proper annealing temperature range for each SSR was determined using a panel of 10 individuals. The single PCR mixtures were composed of 1 U of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA), 1× GoTaq Flexi Buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$, 0.2 mM of dNTP, 0.24 µM of each primer and 16 ng genomic DNA in a final reaction volume of 10 µl. PCR was performed as follows: an initial denaturation at 95°C

for 2 min was followed by 30 cycles of 95°C for 30 s, T_a (provided by primer synthesis information) for 30 s, 72°C for 45 s; and final elongation at 72°C for 30 s. Products were mixed with formamide and GeneScan 500-ROX size standard (Applied Biosystems, Warrington, UK) respectively, according to the manufacturer's recommendations. After 5 min denaturation followed by rapid cooling, PCR products were detected using an ABI 3130*xl* Genetic Analyser (Applied Biosystems, Carlsbad, WA, USA), and the fragment length was estimated through the GeneMapper 3.7 software. These loci were then organized into multiplex sets that maximize the number of loci suitable for simultaneous analysis with no allele overlap between loci. Even with the same dye, overlapping is avoided in order to optimize the genotyping.

Primer concentration, annealing temperature and DNA template concentration were then optimized using 10 individuals. PCR multiplex amplifications were conducted using the Type-it Microsatellite PCR Kit (QIAGEN, Hilden, Germany) in 10 µl reaction volumes containing 5 µl of the Type-it Multiplex PCR Master Mix (2×) (including HotStarTaq® Plus DNA Polymerase, Type-it Microsatellite PCR Buffer with 6 mM $MgCl_2$, and dNTPs), 1µl of primer mix (0.05-0.4 µM), 1 µl of genomic DNA (3-10ng), 1 µl Q-Solution (5×) and 2 µl of RNase-free water. Amplification started with an initial activation step at 95°C for 15 min, followed by 30 cycles with denaturation at 94°C for 30 s, T_a (50-62°C) for 90 s, extension at 72°C for 90 s and final extension at 60°C for 10 min. The PCR products were loaded in the ABI 3130xl Genetic Analyser and analyzed as described in single PCR protocol.

The number of alleles (N_a), polymorphic information content (PIC) and the average non-exclusion probability of each locus in different situation were calculated using Cervus 3.0 (Kalinowski et al., 2007). Allelic Richness (A_r) was estimated with FSTAT 2.9.3 (Goudet, 2001). The genotype data of the progenies in all the 12 single-pair families were pooled together to test the resolve power of the Multiplex PCRs in parentage analysis. The simulation and real parentage assignment were conducted using the likelihood-based approach in CERVUS 3.0. as follows: 10,000 replication cycles, a pool of 24 candidate parents, 100% of the candidate parents sampled and genotyped, a default typing error rate of 1% was used. All correctly allocated offspring were included in the Mendelian inheritance analysis at four EST-SSR multiplex PCRs in this study. The chi-square analysis (with n-1 degrees of freedom, where n = number of phenotypic classes) was used to measure the goodness-of-fit for expected Mendelian segregation ratios (1:1, 1:2:1, and 1:1:1:1) at the 0.01 probability level.

3. Results and discussion

For each single PCR, the range of the suitable annealing temperature was about ± 5°C around the T_a which was provided by primer synthesis information. Therefore we combined the loci according to a common annealing temperature into four optimized sets of multiplex PCRs presented in Table 1, each of them containing three markers. The optimum DNA template quantity was 3ng which resulted in clearly resolved peaks and unambiguous allele calling in all multiplex reactions. Increasing the template quantity produced interaction effects among fluorochromes, and compromised our ability to reliably score genotypes. The genetic diversity and non-exclusion probabilities for each SSR were also shown in Tables 1. The combined power of exclusion was greater than 99.99% across the four panels. The allelic richness per locus ranged from 4 to 16.4, with an average of 11.1. The average PIC was 0.811 from which a high exclusion power was revealed for these Multiplex PCRs in parentage analysis.

The results of Cervus simulations showed that with only two most informative Multiplex PCRs, the total assignment success could be 100% (Fig 1). In practice, the real parentage analysis performed with 12 *C. gigas* single-pair mating families demonstrated that 97% of all offspring were unambiguously assigned to a pair of parents based on the two most informative

multiplex PCRs with only 4% error rate comparing with the real family data, and 100% of the offspring were correctly allocated to their parents when three and four multiplex PCRs were used (Fig 1). The precision of assignment to one correct parental pair depends not only on the number of SSR loci genotyped and their levels of polymorphism but also on the number of potential pairings from which to choose (Matson et al., 2008; Norris et al., 2000). According to simulation, the use of the three multiplex PCRs could also achieve 100% assignment success when the parents increase to 50 pairs, which was recommended by Bentsen and Olesen (2002) to prevent inbreeding and obtain a long-term response in a mass selection program. Actually, in this study, the assignment success rate still sustains at 97% when the parents rise up to 800 pairs with the most polymorphic three multiplex PCRs (Fig 2). Thus, two to three multiplex PCRs developed here should be suitable to perform a successful parentage analysis with lower error rate.

The parental genotypes, observed and expected genotypic frequencies of offspring in each family at each of the four multiplex PCRs were shown in Table 2. SSRs developed from *C. gigas* usually have particularly high frequency null alleles (Li et al., 2003). The presence of null alleles is a classical source of error in parentage assignment with SSRs (Marshall et al., 1998) and it could also bias the estimation of population differentiation (Chapuis and Estoup, 2007). In non-coding genomic SSRs in *C. gigas* null alleles frequencies have been reported from 11% to 22% (Li L et al., 2009; McGoldrick et al., 2000). The frequency of null alleles in EST-SSRs is usually lower than in non-coding genomic SSRs because of lower mutation at splicing sites (Goldstein and Schlotterer, 1999). Yu and Li 2008 reported a null allele frequency of 2.1% of alleles in 20 EST-derived SSRs of *C. gigas*. Among the 12 loci studied here, null alleles were detected for 3 loci (Cgsili39, CGG008, Cgsili6) based on a within-family analysis of Mendelian segregation patterns, 28 of the 576 parental alleles were null alleles, which was 4.9% of the total alleles (12 loci * 24parents *2). The loci showing null alleles were amplified by single PCRs, which confirmed that they were not caused by the mixture of different primers. Of the 144 genotypic ratios examined (12 families × 12 loci), 1 came from crosses between homozygous parents and thus resulted in offspring identical to the parents or with the expected heterozygote genotype (CGE007 in family LOT 1). Eleven genotypic ratios were still not in agreement with Mendelian segregation after accounting for the presence of null alleles (Table 2) which might reflect the impact of selective breeding process.

In conclusion, the results obtained in this study make the four EST-SSR Multiplex PCRs unique tools in studies of parentage assignment, marker assisted breeding, population genetic analysis and linkage maps of *C. gigas*.

4. Acknowledgements

This research has been partially financed by the European Seventh Framework Programme (FP7) through the European Knowledge Based Bio-Economy (KBBE) project REPROSEED (KBBE-3-245119). We also acknowledge the support provided by China Scholarship Council (CSC) to R. Li for her fellowship in Ifremer.

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Table 2. Segregation analysis of microsatellite alleles in *Crassostrea gigas* from twelve full-families.

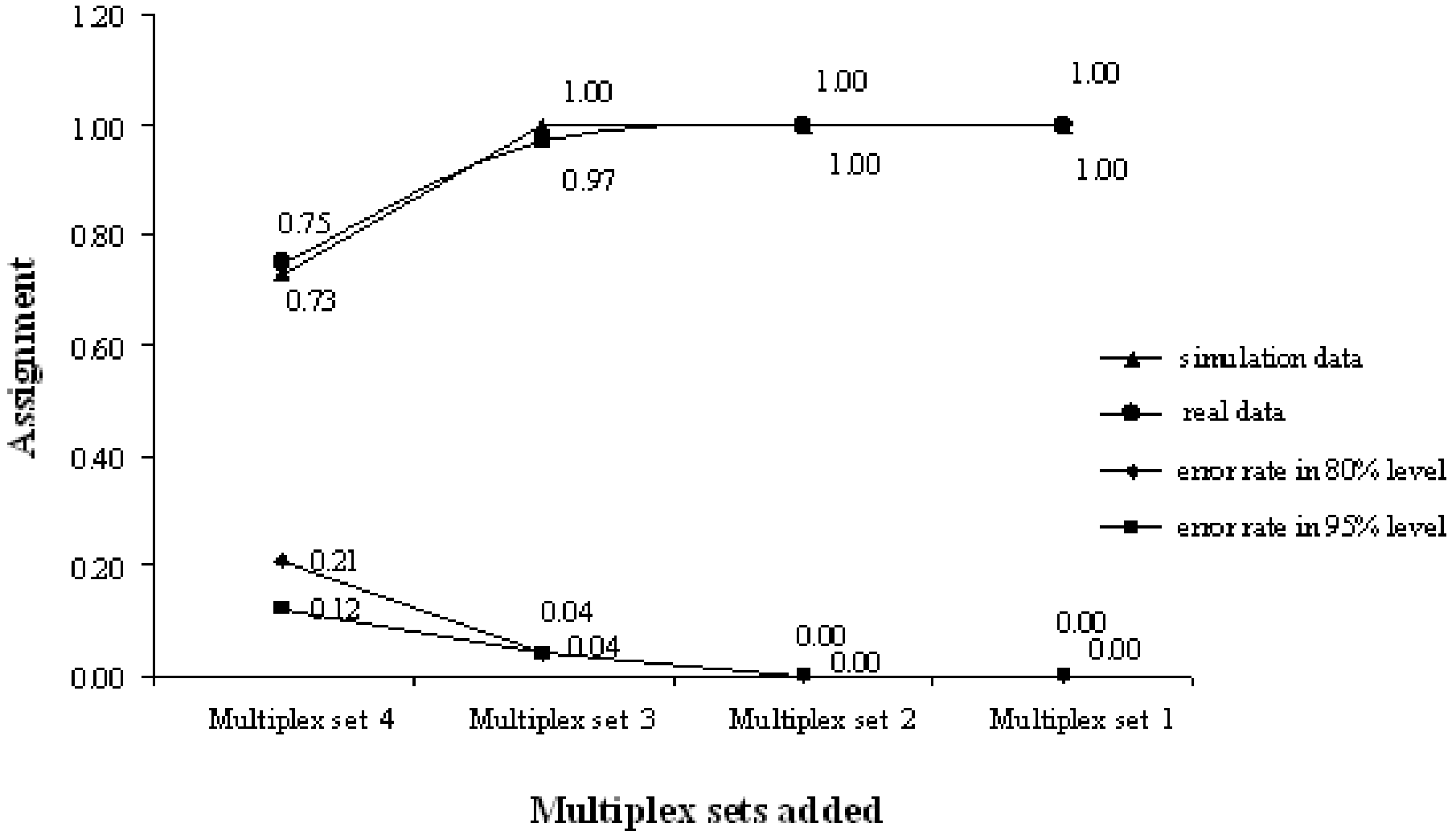
Family	Multiplex	Locus	Dam	Sire	Genotypes of progeny	Expected ratio	Observes ratio	*P* value
LOT 1	Multiplex set 1	CGE007	A/A	A/A	A/A	1	23	-
		Cgsili43	A/B	C/D	A/C:A/D:B/C:B/D	1:1:1:1	4:9:3:7	0.266
		Cgsili46	A/A	B/A	A/B:A/A	1:1	15:8	0.144
	Multiplex set 2	CGE009	A/B	A/C	A/A:A/C:A/B:B/C	1:1:1:1	7:8:2:5	0.282
		AMY	A/B	C/A	A/C:A/A:B/C:B/A	1:1:1:1	9:5:0:6	0.038
		Cgsili44	A/B	C/C	A/C:B/C	1:1	7:14	0.127
	Multiplex set 3	Cgsili39	A/X	B/X	A/B:A/X:X/B:XX	1:1:1:1	20:2:1:0	0.000
		Cgsili50	A/B	B/C	A/B:A/C:B/B:B/C	1:1:1:1	8:6:1:8	0.127
		Cgsili4	A/B	C/D	A/C:A/D:B/C:B/D	1:1:1:1	2:3:11:7	0.032
	Multiplex set 4	CGG008	A/X	B/C	A/B:A/C:X/B:X/C	1:1:1:1	4:4:6:4	0.881
		Cgsili37	A/B	C/B	A/C:A/B:B/C:B/B	1:1:1:1	9:5:7:2	0.199
		Cgsili6	A/B	B/X	A/B:A/X:(B/B + B/X)	1:1:2	9:1:13	0.114
LOT 2	Multiplex set 1	CGE007	B/A	C/A	B/C:B/A:A/C:A/A	1:1:1:1	2:11:0:9	0.001
		Cgsili43	A/E	A/F	A/A:A/F:E/A:E/F	1:1:1:1	6:8:3:6	0.529
		Cgsili46	C/A	C/D	C/C:C/D:C/A:D/A	1:1:1:1	9:3:5:4	0.267
	Multiplex set 2	CGE009	D/A	E/D	D/E:D/D:A/E:A/D	1:1:1:1	6:7:6:4	0.843
		AMY	D/E	F/G	D/F:D/G:E/F:E/G	1:1:1:1	1:1:10:8	0.004
		Cgsili44	B/C	B/B	B/B:B/C	1:1	14:8	0.201
	Multiplex set 3	Cgsili39	B/X	A/X	B/A:B/X:X/A:X/X	1:1:1:1	8:1:11:3	0.012
		Cgsili50	A/D	E/F	A/E:A/F:D/E:D/F	1:1:1:1	1:7:3:12	0.006
		Cgsili4	E/B	B/F	E/B:E/F:B/B:B/F	1:1:1:1	2:7:7:7	0.353
	Multiplex set 4	CGG008	D/B	E/X	D/E:D/X:B/E:B/X	1:1:1:1	5:9:2:7	0.199
		Cgsili37	D/E	F/G	D/F:D/G:E/F:E/G	1:1:1:1	9:7:3:4	0.266
		Cgsili6	C/D	E/C	C/E:C/C:D/E:D/C	1:1:1:1	7:3:7:5	0.572
LOT 3	Multiplex set 1	CGE007	B/D	E/A	B/E:B/A:D/E:D/A	1:1:1:1	7:6:7:2	0.378
		Cgsili43	G/H	A/I	G/A:G/I:H/A:H/I	1:1:1:1	4:7:6:4	0.733
		Cgsili46	D/B	E/F	D/E:D/F:B/E:B/F	1:1:1:1	4:4:7:7	0.651
	Multiplex set 2	CGE009	F/C	E/F	F/E:F/F:C/E:C/F	1:1:1:1	6:8:2:6	0.327
		AMY	E/H	A/I	E/A:E/I:H/A:H/I	1:1:1:1	4:3:4:10	0.119
		Cgsili44	B/D	D/D	B/D:D/D	1:1	10:11	0.827
	Multiplex set 3	Cgsili39	A/C	D/X	A/D:A/X:C/D:C/X	1:1:1:1	1:7:5:7	0.187
		Cgsili50	A/D	B/B	A/B:D/B	1:1	10:8	0.637
		Cgsili4	G/H	B/B	G/B:H/B	1:1	11:11	1.000
	Multiplex set 4	CGG008	A/F	D/B	A/D:A/B:F/D:F/B	1:1:1:1	5:6:8:3	0.500
		Cgsili37	H/B	I/J	H/I:H/J:B/I:B/J	1:1:1:1	3:9:6:4	0.282
		Cgsili6	F/X	F/X	(F/F + F/X):X/X	3:1	19:4	0.399
LOT 4	Multiplex set 1	CGE007	A/A	E/F	A/E:A/F	1:1	14:9	0.297
		Cgsili43	A/J	H/J	A/H:A/J:J/H:J/J	1:1:1:1	6:4:5:6	0.914
		Cgsili46	C/A	C/A	C/C:C/A:A/A	1:2:1	6:8:8	0.572
	Multiplex set 2	CGE009	E/C	F/C	E/F:E/C:F/C:C/C	1:1:1:1	5:7:8:3	0.464
		AMY	J/K	H/K	J/H:J/K:K/H:K/K	1:1:1:1	6:3:8:2	0.188
		Cgsili44	C/C	A/C	C/A:C/C	1:1	16:6	0.033
	Multiplex set 3	Cgsili39	D/X	E/X	D/E:D/X:X/E:X/X	1:1:1:1	4:6:8:2	0.261
		Cgsili50	B/C	B/D	B/B:B/D:C/B:C/D	1:1:1:1	4:5:8:5	0.651
		Cgsili4	I/B	G/I	I/G:I/I:B/G:B/I	1:1:1:1	6:7:5:4	0.823
	Multiplex set 4	CGG008	G/X	B/G	G/B:(G/G + G/X):X/B	1:2:1	2:16:2	0.066
		Cgsili37	A/B	A/E	A/A:A/E:B/A:B/E	1:1:1:1	8:3:7:4	0.378
		Cgsili6	B/X	G/H	B/G:B/H:X/G:X/H	1:1:1:1	4:2:9:4	0.131
LOT 5	Multiplex set 1	CGE007	G/B	G/A	G/G:G/A:B/G:B/A	1:1:1:1	6:6:3:8	0.529
		Cgsili43	G/A	C/D	G/C:G/D:A/C:A/D	1:1:1:1	4:5:10:3	0.153
		Cgsili46	A/G	A/H	A/A:A/H:G/A:G/H	1:1:1:1	5:5:5:8	0.759
	Multiplex set 2	CGE009	D/B	E/D	D/E:D/D:B/E:B/D	1:1:1:1	6:6:6:5	0.988
		AMY	L/A	G/I	L/G:L/I:A/G:A/I	1:1:1:1	5:4:6:7	0.823
		Cgsili44	A/D	C/C	A/C:D/C	1:1	7:16	0.061
	Multiplex set 3	Cgsili39	F/G	A/X	F/A:F/X:G/A:G/X	1:1:1:1	8:6:3:5	0.500
		Cgsili50	B/D	B/C	B/B:B/C:D/B:D/C	1:1:1:1	3:3:9:8	0.148
		Cgsili4	B/J	C/K	B/C:B/K:J/C:J/K	1:1:1:1	5:8:6:4	0.677
	Multiplex set 4	CGG008	H/X	B/C	H/B:H/C:X/B:X/C	1:1:1:1	5:4:4:10	0.23
		Cgsili37	C/B	B/J	C/B:C/J:B/B:B/J	1:1:1:1	4:7:5:7	0.759
		Cgsili6	E/X	X/X	E/X:X/X	1:1	10:13	0.532
LOT 6	Multiplex set 1	CGE007	A/A	F/A	A/F:A/A	1:1	14:9	0.297
		Cgsili43	H/B	A/J	H/A:H/J:B/A:B/J	1:1:1:1	8:7:1:7	0.148
		Cgsili46	C/B	A/A	C/A:B/A	1:1	9:14	0.297
	Multiplex set 2	CGE009	D/A	D/C	D/D:D/C:A/D:A/C	1:1:1:1	4:7:6:6	0.843
		AMY	C/M	E/K	C/E:C/K:M/E:M/K	1:1:1:1	7:4:2:9	0.153
		Cgsili44	B/D	A/B	B/A:B/B:D/A:D/B	1:1:1:1	5:6:2:9	0.208
	Multiplex set 3	Cgsili39	B/D	E/F	B/E:B/F:D/E:D/F	1:1:1:1	6:3:10:4	0.172
		Cgsili50	B/G	A/E	B/A:B/E:G/A:G/E	1:1:1:1	8:1:11:3	0.012
		Cgsili4	G/B	E/B	G/E:G/B:B/E:B/B	1:1:1:1	8:7:5:2	0.282
	Multiplex set 4	CGG008	I/B	A/G	I/A:I/G:B/A:B/G	1:1:1:1	6:7:5:5	0.924
		Cgsili37	K/E	L/B	K/L:K/B:E/L:E/B	1:1:1:1	9:4:6:4	0.405
		Cgsili6	G/I	B/J	G/B:G/J:I/B:I/J	1:1:1:1	8:10:3:2	0.051

Table 2 (*continued*)

Family	Multiplex	Locus	Dam	Sire	Genotypes of progeny	Expected ratio	Observes ratio	*P* value
LOT 7	Multiplex set 1	CGE007	H/A	A/A	H/A:A/A	1:1	16:7	0.061
		Cgsili43	A/J	G/K	A/G:A/K:J/G:J/K	1:1:1:1	3:8:3:9	0.148
		Cgsili46	A/H	A/G	A/A:A/G:H/A:H/G	1:1:1:1	2:9:3:9	0.059
	Multiplex set 2	CGE009	A/C	A/B	A/A:A/B:C/A:C/B	1:1:1:1	4:5:7:7	0.759
		AMY	N/O	A/K	N/A:N/K:O/A:O/K	1:1:1:1	3:5:5:9	0.327
		Cgsili44	B/D	D/C	B/D:B/C:D/D:D/C	1:1:1:1	4:6:5:8	0.677
	Multiplex set 3	Cgsili39	A/X	A/G	(A/A + X/A):A/G:X/G	2:1:1	10:4:9	0.464
		Cgsili50	A/B	A/B	A/A:A/B:B/B	1:2:1	4:10:9	0.464
		Cgsili4	I/B	J/L	I/J:I/L:B/J:B/L	1:1:1:1	6:6:5:6	0.989
	Multiplex set 4	CGG008	X/X	E/A	X/E:X/A	1:1	12:10	0.670
		Cgsili37	I/B	B/M	I/B:I/M:B/B:B/M	1:1:1:1	7:9:7:0	0.043
		Cgsili6	E/B	C/D	E/C:E/D:B/C:B/D	1:1:1:1	8:8:4:3	0.307
LOT 8	Multiplex set 1	CGE007	E/A	F/B	E/F:E/B:A/F:A/B	1:1:1:1	8:4:4:6	0.572
		Cgsili43	A/L	C/C	A/C:L/C	1:1	9:13	0.394
		Cgsili46	A/A	B/A	A/B:A/A	1:1	12:10	0.670
	Multiplex set 2	CGE009	A/C	D/B	A/D:A/B:C/D:C/B	1:1:1:1	9:2:7:5	0.199
		AMY	E/G	A/O	E/A:E/O:G/A:G/O	1:1:1:1	3:9:4:7	0.266
		Cgsili44	C/C	A/D	C/A:C/D	1:1	8:14	0.201
	Multiplex set 3	Cgsili39	A/C	C/X	A/C:A/X:(C/C + C/X)	1:1:2	0:7:15	0.061
		Cgsili50	C/D	B/B	C/B:D/B	1:1	7:15	0.088
		Cgsili4	B/J	G/G	B/G:J/G	1:1	3:11	0.033
	Multiplex set 4	CGG008	E/X	H/F	E/H:E/F:X/H:X/F	1:1:1:1	9:4:6:4	0.405
		Cgsili37	N/O	H/L	N/H:N/L:O/H:O/L	1:1:1:1	1:5:4:13	0.003
		Cgsili6	X/X	B/X	X/B:X/X	1:1	5:18	0.007
LOT 9	Multiplex set 1	CGE007	B/A	G/B	B/G:B/B:A/G:A/B	1:1:1:1	7:2:10:4	0.094
		Cgsili43	A/C	G/A	A/G:A/A:C/G:C/A	1:1:1:1	3:2:5:10	0.055
		Cgsili46	B/A	B/E	B/B:B/E:A/B:A/E	1:1:1:1	5:10:4:3	0.153
	Multiplex set 2	CGE009	D/G	D/F	D/D:D/F:G/D:G/F	1:1:1:1	3:5:9:5	0.327
		AMY	P/K	A/H	P/A:P/H:K/A:K/H	1:1:1:1	2:3:7:5	0.325
		Cgsili44	B/D	A/D	B/A:B/D:D/A:D/D	1:1:1:1	7:5:4:7	0.759
	Multiplex set 3	Cgsili39	A/X	A/G	(A/A + X/A) :A/G:X/G	2:1:1	4:12:7	0.008
		Cgsili50	B/B	A/C	B/A:B/C	1:1	13:10	0.532
		Cgsili4	B/K	B/J	B/B:B/J:K/B:K/J	1:1:1:1	2:4:9:5	0.158
	Multiplex set 4	CGG008	H/I	E/H	H/E:H/H:I/E:I/H	1:1:1:1	8:2:6:3	0.188
		Cgsili37	B/N	C/B	B/C:B/B:N/C:N/B	1:1:1:1	8:4:4:5	0.563
		Cgsili6	A/B	K/C	A/K:A/C:B/K:B/C	1:1:1:1	4:8:4:4	0.494
LOT 10	Multiplex set 1	CGE007	I/A	E/A	I/E:I/A:A/E:A/A	1:1:1:1	3:9:3:8	0.148
		Cgsili43	M/A	G/N	M/G:M/N:A/G:A/N	1:1:1:1	10:8:1:3	0.022
		Cgsili46	C/A	B/G	C/B:C/G:A/B:A/G	1:1:1:1	3:5:7:8	0.464
	Multiplex set 2	CGE009	D/F	H/A	D/H:D/A:F/H:F/A	1:1:1:1	6:7:5:5	0.924
		AMY	H/K	A/K	H/A:H/K:K/A:K/K	1:1:1:1	4:8:7:4	0.529
		Cgsili44	B/B	A/C	B/A:B/C	1:1	15:8	0.144
	Multiplex set 3	Cgsili39	A/H	F/G	A/F:A/G:H/F:H/G	1:1:1:1	2:12:2:7	0.008
		Cgsili50	C/H	A/B	C/A:C/B:H/A:H/B	1:1:1:1	10:4:4:5	0.230
		Cgsili4	M/N	J/O	M/J:M/O:N/J:N/O	1:1:1:1	8:8:2:3	0.119
	Multiplex set 4	CGG008	I/F	I/G	I/I:I/G:F/I:F/G	1:1:1:1	7:5:5:6	0.924
		Cgsili37	F/M	D/N	F/D:F/N:M/D:M/N	1:1:1:1	4:7:4:8	0.529
		Cgsili6	G/X	B/X	G/B:G/X:X/B:X/X	1:1:1:1	4:15:4:0	0.000
LOT11	Multiplex set 1	CGE007	G/A	G/A	G/G:G/A:A/A	1:2:1	7:10:6	0.924
		Cgsili43	H/D	O/C	H/O:H/C:D/O:D/C	1:1:1:1	6:5:5:7	0.924
		Cgsili46	C/G	G/E	C/G:C/E:G/G:G/E	1:1:1:1	7:9:4:3	0.266
	Multiplex set 2	CGE009	A/C	C/B	A/C:A/B:C/C:C/B	1:1:1:1	6:7:6:4	0.843
		AMY	N/Q	A/K	N/A:N/K:Q/A:Q/K	1:1:1:1	6:6:3:4	0.701
		Cgsili44	A/C	B/D	A/B:A/D:C/B:C/D	1:1:1:1	6:6:7:4	0.843
	Multiplex set 3	Cgsili39	E/E	E/C	E/E:E/C	1:1	23:0	0.000
		Cgsili50	F/H	A/B	F/A:F/B:H/A:H/B	1:1:1:1	7:3:3:10	0.110
		Cgsili4	P/C	O/O	P/O:C/O	1:1	11:12	0.835
	Multiplex set 4	CGG008	F/C	I/I	F/I:C/I	1:1	12:11	0.835
		Cgsili37	C/N	H/N	C/H:C/N:N/H:N/N	1:1:1:1	3:10:3:7	0.110
		Cgsili6	L/I	M/B	L/M:L/B:I/M:I/B	1:1:1:1	10:5:5:3	0.199
LOT 12	Multiplex set 1	CGE007	J/J	E/A	J/E:J/A	1:1	7:5	0.564
		Cgsili43	B/P	K/J	B/K:B/J:P/K:P/J	1:1:1:1	6:3:9:5	0.353
		Cgsili46	I/A	A/G	I/A:I/G:A/A:A/G	1:1:1:1	8:3:4:8	0.307
	Multiplex set 2	CGE009	E/C	H/A	E/H:E/A:C/H:C/A	1:1:1:1	2:8:8:5	0.230
		AMY	Q/O	C/G	Q/C:Q/G:O/C:O/G	1:1:1:1	6:6:8:2	0.327
		Cgsili44	C/C	B/D	C/B:C/D	1:1	9:13	0.394
	Multiplex set 3	Cgsili39	C/E	D/E	C/D:C/E:E/D:E/E	1:1:1:1	8:15:0:0	0.000
		Cgsili50	A/C	A/D	A/A:A/D:C/A:C/D	1:1:1:1	7:4:5:6	0.823
		Cgsili4	B/Q	I/L	B/I:B/L:Q/I:Q/L	1:1:1:1	2:7:5:9	0.199
	Multiplex set 4	CGG008	G/C	F/X	G/F:G/X:C/F:C/X	1:1:1:1	3:1:11:7	0.013
		Cgsili37	F/P	D/B	F/D:F/B:P/D:P/B	1:1:1:1	7:8:5:3	0.464
		Cgsili6	N/B	C/G	N/C:N/G:B/C:B/G	1:1:1:1	4:4:5:9	0.378

Genotypic ratios that are not in agreement with Mendelian segregation.

Figures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Assignment
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1.00
0.75
0.73
0.21
0.12
0.04
0.04
0.00
0.00
0.00
0.00
simulation data
real data
error rate in 80% level
error rate in 95% level
Multiplex set 4
Multiplex set 3
Multiplex set 2
Multiplex set 1
Multiplex sets added

Fig. 1. Cumulative assignment success rates of simulated and real genotype data in a strict level of 95% confidence interval. The error rates were calculated in 95% and 80% confidence interval comparing with the family data. The multiplex sets were added from the most to the least average PIC value.

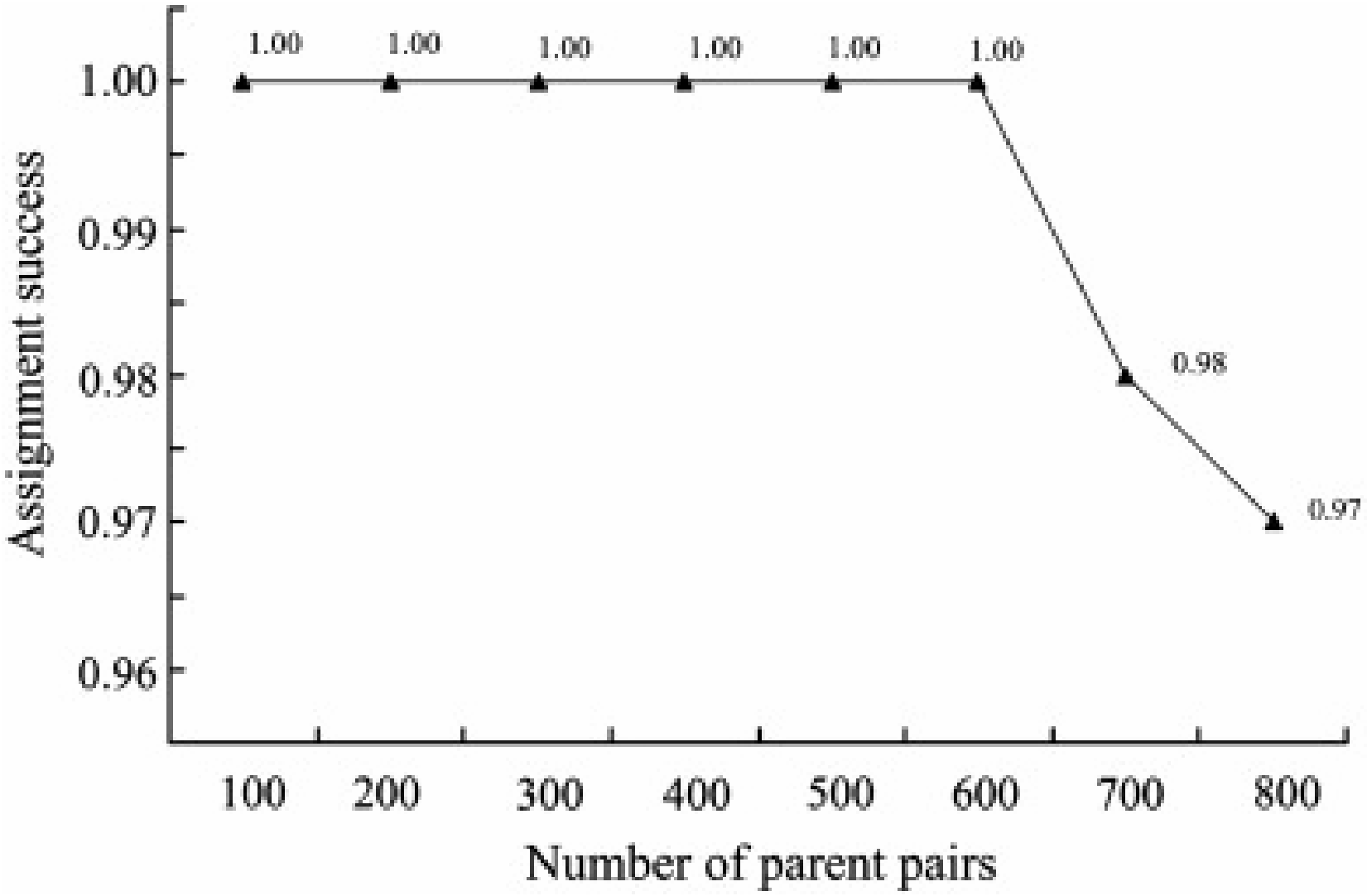

Fig. 2. Simulated assignment success rates of 10,000 offspring in parentage under a number of candidate parents from 100 to 800 with the most Polymorphic three multiplex PCRs in a strict level of 95% confidence interval.

Table 1. Characteristics of the four EST-SSR multiplex PCRs in *Crassostrea gigas* .

Locus	Repeat motif	Primer sequence (5′–3′)	T_a ( )	Size range (bp)	Concentration in multiplex-PCR (μM)	N_a	A_r	PCI	NE-1P[a]	NE-2P[b]	NE-PP[c]	GenBank Accession no.
PCR multiplex set 1			58									
CGE007	$(TA)_7$	**F:** TTTCCCCTGAGAAGACCC **R: NED**-AACCAAATCCATTCAACATAAC		96-142	0,1	10	9,7	0,702	0,650	0,462	0,251	BQ427084
Cgsili43	$(GA)_{10}$	**F: HEX**-AAATGCTGCAGAAATAATCC **R:** AGATGGCTACAGTGAAATGG		210-348	0,3	16	15,4	0,874	0,370	0,227	0,076	AM854072
Cgsili46	$(TG)_5A(GT)_7$	**F: FAM**-CATGACAATCGAGTCCATAA **R:** CATGGTGGAGAAAGAGTTGT		165-211	0,09	9	8,8	0,753	0,588	0,406	0,210	AM856490
PCR multiplex set 2			52									
CGE009	$(AG)_7$	**F:** TTCGTTGAAGGTGACAAGTG **R: NED**-GCATTTTGGGATGAACAGA		114-128	0,1	8	7,9	0,814	0,501	0,330	0,157	CX068958
AMY	$(TC)_{37}$	**F: HEX**-ACCGGTATTGCCCGAGTTACAA **R:** AGTTAGGCATCCCCCATTGTTC		199-369	0,2	17	16,4	0,892	0,331	0,198	0,061	Y08370
Cgsili44	$(AG)_7AAA(GA)_4$	**F: FAM**-TGGCATTTCATGGTTAATTT **R:** TGTTGTATGAAATGTCGGAA		349-355	0,1	4	4,0	0,690	0,685	0,511	0,337	AM858556
PCR multiplex set 3			58									
Cgsili39	$(AG)_{13}$	**F: FAM**-GACCATACAGCTCTGTCCAT **R:** GCTACTGAATGAGAATGGCT		355-383	0,09	8	7,9	0,801	0,519	0,345	0,164	AM854746
Cgsili50	$(CA)_{10}$	**F: FAM**-CTATCTGAGCACGCTTCTCT **R:** TCTCTGTCAGATGATCTCAGG		201-233	0,09	8	7,9	0,750	0,595	0,416	0,227	AM865904
Cgsili4	$(AG)_{26}$	**F: HEX**-GGTGCAGTAGTTGGAAACAT **R:** TCACATTTAACTAGCGCTCTC		227-349	0,24	17	16,3	0,860	0,396	0,246	0,085	AM854894
PCR multiplex set 4			58									
CGG008	$(AG)_{20}$	**F:** TCTCCTCTACCCCGACAG **R: NED**-GTGATGAACAAACCACCAAC		181-253	0,4	9	9,0	0,870	0,392	0,242	0,091	AJ579915
Cgsili37	$(TC)_{15}$	**F: FAM**-TTGCTGGTTGTGATGAATAG **R:** ATATCTGGCCTAACATGTGC		159-293	0,15	16	15,6	0,890	0,335	0,201	0,061	BQ427164
Cgsili6	$(GA)_{26}$	**F: HEX**-ATGAACGTCCAAGTTCAGAC **R:** ACACATTTCCTTATAAAGCC		270-442	0,2	14	14,0	0,834	0,449	0,287	0,112	AM854296

Number of alleles (N_a), allelic richness (A_r), polymorphic information content (PIC), and average non-exclusion probability of each locus surveyed in the 24 *C. gigas* parents.

The fluorescent labels were indicated by bold letters in front of the primer sequences.

[a] Average non-exclusion probability for one candidate parent.

[b] Average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex.

[c] Average non-exclusion probability for a candidate parent pair.