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Accuracy of short sequence repeats on single-parent parentage identification in Chinese shrimp *Fenneropenaeus chinensis*

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ABSTRACT: Based on the parentage identification results using 4 polymorphic short sequence repeats (SSRs) with both parents known, we assessed the accuracy of parentage identification for a single known parent using the same 4 SSRs and 4 other additional SSRs for 2507 Chinese shrimp *Fenneropenaeus chinensis* recaptured from Jiaozhou Bay in a 'molecular marker recapture' program. We detected a high variability in the 4 SSR loci EN0033, RS0622, FCKR002, and FCKR013. The 4 polymorphic loci produced a ≥99.99% cumulative exclusion probability for both known parents. We identified 8 parentage relationships with a logarithm (base 10) of odds (LOD) score of ≥3.0. Similar results were obtained when the 4 additional SSR loci were included. The cumulative exclusion probability decreased to 99.42% for a single known parent using the old 4 SSR loci, which enabled detection of 30 and 44 parentage relationships with a ≥3.0 LOD score for known paternity and maternity, respectively. Inclusion of the additional 4 SSR loci raised, the cumulative exclusion probability to ≥99.99%. However, the number of parentage relationships detected with an LOD score of ≥3.0 was reduced to 8 for the groups with known paternity or maternity, similar to the result obtained with both parents known. In this study, the accuracy of single-parent parentage identification using SSR loci was confirmed. Authentic parentage identification with a single known parent should incorporate both the cumulative exclusion probability and LOD score. Our research provides an alternative method for parentage identification in Chinese shrimp.

KEY WORDS: *Fenneropenaeus chinensis* · Microsatellites · Parentage identification

INTRODUCTION

Chinese shrimp *Fenneropenaeus chinensis* are primarily distributed over the Yellow and Bohai Seas. It is one of the most important shrimp species, with a high commercial value in marine aquaculture. It is nutrient-rich and grows rapidly — an advantage in meat production (Deng et al. 1990). In recent years, advances in genetic breeding have led to resource recovery (hatchery release) for Chinese shrimp. Different types of physical tagging, including eye-tags, visible implant elastomers (VIEs), and coded wire tags (CWTs), as well as parentage identification techniques based on molecular fingerprinting have been developed. Parentage identification based on molecular fingerprinting has wide application in forensic examination, livestock industry, and genetic breeding (Fisher et al. 2009, Costa et al. 2012). It also been used in selective breeding programs in aquatic species, where physical tagging is not always the optimal choice for individual or family identification. Aquatic species undergo ecdysis several times in their life, and larvae are usually too small to incorporate any kind of tag. Physical tags, such as VIEs, have been used to discriminate different families in traditional breeding practices (Godin et al. 1996, Meehan et al. 2003, Luo et al. 2008). However, physical tagging is not an ideal approach for aquatic animals in genetic breeding programs, marker-recapture assessments, hatchery release, or other fields. Most

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physical tagging methods used for aquatic animals, such as shrimp, fish, and crab, either hurt the tagged individuals or are impractical for large-scale applications. Further, the tagging process is labor-intensive (Soula et al. 2007).

The power and accuracy of molecular markers, especially short sequence repeats (SSRs), for individual identification and pedigree tracing have been validated in animals. Bai et al. (2010) successfully traced the pedigree of 61 blue foxes *Alopex lagopus* based on 15 microsatellite markers and GeneScan technology. Qian et al. (2010) demonstrated the accuracy of 10 microsatellite loci in estimating genetic relationships in standard triplet and single parent investigations of Wenshan cattle *Bos taurus*. Compared with terrestrial species, most aquatic animals are fecund. The filial origin of the larvae is not easy to trace using a physical tag, and thus it is difficult to meet the requirements of breeding programs or marker-recapture assessment in hatchery release (Li et al. 2012). Parentage identification based on molecular markers is a promising approach. In aquatic animals, SSR markers have also been widely used in genetic diversity studies, individual or family tracing, and linkage mapping (Hulak et al. 2010, Ruan et al. 2010, Arabnezhad et al. 2011, Borrell et al. 2011). Given the abundance of allele variability, SSR loci are helpful for individual identification and pedigree tracing. The parentage assignment efficiency exceeded 92.9% for pedigree identification using 5 microsatellites in 215 Chinese shrimp offspring (*Fenneropenaeus chinensis*) (Dong et al. 2006). In the Kuruma prawn *Penaeus japonicus*, offspring were assigned to 13 families using 7 high-polymorphism SSR loci (Jerry et al. 2006). One hundred and sixty progenies of eastern oysters *Crassostrea virginica* Gmelin from a putative pool of 81 full-sib families were successfully genotyped using 16-microsatellite multiplex PCR and parentage was unambiguously assigned (Wang et al. 2010).

Accurate pedigree analysis is important in modern animal breeding programs, especially for precise estimation of genetic parameters and additive genetic variances between individuals (Falconer & Mackay 1996, Lynch & Walsh 1997). However, in most aquatic animals, the paternities are not often available due to specific reproductive behaviors. For example, the wild Chinese shrimp completes the mating progress during overwintering migration in autumn, and most of the male individuals die thereafter. The next spring, the shrimp captured for production of larvae only include mature females bearing the spermatophore. In certain cases, such as in captive species with natural mating in groups, pedigree reconstruction is necessary, e.g. in genetic parameter estimation for a selective breeding program. In such instances, single paternity identification based on molecular markers would be useful.

In the present study, a molecular marker recapture assessment of hatchery release activity was introduced, where 6 cultivated full-sib families were mixed with a hatchery release stock of *Fenneropenaeus chinensis* and released into Jiaozhou Bay in China in spring 2012. Using genotyping with 8 SSR loci, we completed the parentage identification. We confirmed that 8 shrimps were from the full-sib families of the 2507 shrimp recaptured in autumn. We then analyzed single-parent parentage relationships, and evaluated for accuracy when both the parents were known. This study assessed the accuracy of single-parent identification and provides a reference for single-parent investigations in complex cases, using microsatellite markers.

MATERIALS AND METHODS

Sample collection

In spring 2012, six full-sib families of *Fenneropenaeus chinensis* were cultivated in the Aquatic Genetic Breeding Center of the Yellow Sea Fisheries Research Institute. The 6 families were referenced as families A to F. The paternity for family F was unavailable due to quality issues of sample preservation. On 22 May, 3×10^5 postlarval shrimps (stage P10) were released into Jiaozhou Bay. In August 2012, a total of 2507 individuals were recaptured near the area of release before they initiated overwintering migration. DNA was extracted from muscle tissue inside the swimming legs of all the recaptured shrimps, and the same tissue from the 11 parents of the 6 full-sib families was also dissected for DNA preparation.

DNA extraction and multiplex SSR-PCR

Genomic DNA was isolated following the standard ammonium-acetate method with minor variations (Wang 2008). Pure DNA was dissolved in double-distilled water to obtain a concentration of 50 ng μl^{-1}, as determined by a GeneQuant spectrophotometer (Amersham Biosciences). The quality of the isolated DNA was determined using agarose gel electrophoresis. DNA solutions were stored at −20°C. A

single set of low-temperature groups (LTGs) for the quadruple SSR-PCR system containing 4 SSR loci with fluorescently labeled primers in *Fenneropenaeus chinensis* was used for genotyping the recaptured shrimp and 11 parents. A high-temperature group (HTG) in the quadruple SSR-PCR system was used to confirm the candidate parentage relationships (Li et al. 2012). Primer details are shown in Table 1. The forward primer for each primer pair from 2 quadruple SSR-PCR sets was labeled with 4 different fluorescent dyes (Applied Biosystems): PET, VIC, 6-FAM, and NED, respectively. The quadruple SSR-PCR reaction components and procedure were adapted from Li et al. (2012).

Allele detection using PCR

An ABI 3130 automatic Genetic Analyzer (Applied Biosystems) was used to separate the PCR products. Alleles from SSR loci were sized with GeneScan TM-500 LIZ Size Standard (Applied Biosystems) and scored using GeneMapper™ v4.1 (Applied Biosystems).

Parentage identification

First, the 4 SSR loci in the LTG PCR system were genotyped for the 2507 recaptured shrimp. Genetic diversity indices, including allele frequencies, expected heterozygosity (H_e), observed heterozygosity (H_o), polymorphism information content (PIC), Hardy-Weinberg equilibrium (HWE), and average non-exclusion probability, were calculated using CERVUS 3.0 (Marshall et al. 1998, Kalinowskiet al. 2007). The power of each locus and cumulative exclusion probabilities for loci with a single known parent were both referenced as E-2P probabilities. The corresponding samples with both parents known were referenced as E-PP probabilities. CERVUS 3.0 assigned the recaptured shrimp to each candidate parent pair (only maternity was available in family F) using locus-by-locus logarithm (base 10) of odds (LOD) scores. For each assignment, if the 4 SSR loci matched and the LOD score was ≥3.0, it was a true parentage relationship. The identified parentage relationships were then validated using an additional 4 SSR loci in the HTG PCR system. Individuals in both quadruple PCR system groups were determined using an 'inner marker'. For the single-parent identification analysis, a similar procedure was adopted, and the same genotype data were used. However, results were generated for only a single known parent (only paternity or maternity available). The accuracy of the parentage identification for samples with 1 known parent was then evaluated using the results from the samples with 2 identified parents.

Table 1. *Fenneropenaeus chinensis*. Details of 8 primer pairs (F: forward, R: reverse) and 2 groups (HTG: high-temperature group, LTG: low-temperature group) of the quadruple short sequence repeat (SSR)-PCR system used. 6-FAM, VIC, NED, and PET are fluorescent dyes

Quadruple PCR set	Locus name	GenBank acc. no.	Annealing temp. (°C)	Primer sequences (5′–3′) labeled with fluorescent dye
HTG	EN0033	AY132813	64	**F: 6-FAM-**CCT TGA CAC GGC ATT GAT TGG **R:** TAC GTT GTG CAA ACG CCA AGC
	RS0622	AY132778	66	**F: VIC-**TCA GTC CGT AGT TCA TAC TTG G **R:** CAC ATG CCT TTG TGT GAA AAC G
	FCKR002	JQ650349	60	**F: NED-**CTC AAC CCT CAC CTC AGG AAC A **R:** AAT TGT GGA GGC GAC TAA GTT C
	FCKR013	JQ650353	61	**F: PET-**GCA CAT ATA AGC ACA AAC GCT C **R:** CTC TCT CGC AAT CTC TCC AAC T
LTG	RS1101	AY132811	52	**F: 6-FAM-**CGA GTG GCA GCG AGT CCT **R:** TAT TCC CAC GCT CTT GTC
	FCKR005	JQ650350	50	**F: VIC-**CAT CGA ATC TAA GAG CTG GAA T **R:** TTT GTT TGT GAA TAA TGT GTG T
	FCKR007	JQ650351	49	**F: NED-**CGA AAT AAG TTA AAT GAA AAA A **R:** CAA CAT AAG ACT CAC GAG ACA G
	FCKR009	JQ650352	52	**F: PET-**GCA CGA AAA CAC ATT AGT AGG A **R:** ATA TCT GGA ATG GCA AAG AGT C

RESULTS

Genetic variability of 8 SSR loci

Based on the genotyping data for the 2507 recaptured samples, the genetic variability of 4 SSR loci in LTG is summarized in Table 2. One-hundred and twenty-nine alleles for the 4 SSR loci were detected, with an average value of 32.25 alleles per locus. The PIC value per locus ranged from 0.765 to 0.955, with an average value of 0.8943, which indicated a higher level of polymorphism (PIC > 0.5). The average H_e was greater than the average H_o for each of the 4 loci, which indicated a loss of heterozygosity.

Exclusion probability of the SSR locus

E-2P (one parent known) for each locus ranged from ~60.0% to 91.4%, and the cumulative exclusion probability for each of the 4 loci was 99.42% with only 1 known parent (Table 2). Further, E-PP (both parents known) for each locus increased and ranged from 78.3 to 98.7%, and the cumulative exclusion probability of each of the 4 loci increased to 99.99% with both parents known.

Individual parentage identification with a single known parent

The genotyping data for the 2507 recaptured samples and 4 SSR loci in the LTG PCR system were first used to identify the 'inner marker' individuals. In the 'Parentage analysis', CERVUS 3.0 assigned each sample to candidate parents or a single parent using locus-by-locus likelihood scores (Table 3). Results showed that only 4 SSR loci in the LTG were sufficient to identify the 'inner marker' individuals with both parents known for a cumulative exclusion probability of 99.99% (or greater), which was equivalent to using 8 SSR loci in the parentage identification with both parents known. For a single-parent parentage analysis, the number of identified parentages with 4 SSR loci exceeded the number where both parents were identified. In fact, 30 and 44 parentage relationships with only the paternity or maternity known respectively were detected with an LOD score of ≥3.0 (Tables 3 & 4). However, when the genotype information for each of the 8 SSR loci were included, the number of parentage relationships detected with only the paternity known decreased from 30 to 8, and the number detected with only the maternity known decreased from 44 to 8. As dis-

Table 2. *Fenneropenaeus chinensis*. Genetic variability of 4 short sequence repeat (SSR) loci. N_a: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; PIC: polymorphic information content; E-1P, E-2P, and E-PP: probabilities of exclusion based on the genotype of no parent known, 1 parent known, and both parents known, respectively; HWE: Hardy-Weinberg equilibrium; nd: not done. ***Significant at the 0.1% level

Locus	Allele size (bp)	N_a	Heterozygosity		PIC	Power of exclusion			HWE
			H_o	H_e		E-1P	E-2P	E-PP	
RS1101	411–580	19	0.718	0.795	0.765	0.423	0.6	0.783	***
FCKR005	215–316	39	0.628	0.95	0.947	0.816	0.898	0.982	nd
FCKR007	189–333	46	0.444	0.957	0.955	0.842	0.914	0.987	nd
FCKR009	162–228	25	0.425	0.915	0.909	0.712	0.831	0.955	nd
Mean N_a per locus								32.25	
Mean H_e								0.9044	
Mean H_o								0.5538	
Mean PIC								0.8943	
Combined exclusion probability (E-1P)								0.98916209	
Combined exclusion probability (E-2P)								0.99420780	
Combined exclusion probability (E-PP)								0.99999765	

Table 3. *Fenneropenaeus chinensis*. Parentage identification based on 4 or 8 short sequence repeat (SSR) loci with both parents or a single parent known. All 6 families were full-sib families. LTG: low-temperature group, na: not available

	Both parents known						Paternity known						Maternity known					
Family	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
4 SSR loci in LTG	3	2	2	0	1	na	9	9	9	2	1	na	9	3	16	5	3	8
All 8 SSR loci	3	2	2	0	1	na	3	2	2	0	1	na	3	2	2	0	1	0

played in Table 4, when the additional 4 SSR loci in the HTG were included in the single-parent parentage analysis, the LOD score for most of the identified parentage relationships decreased to <3.0. The remaining individuals with an LOD value of ≥3.0 from a single-parent parentage analysis with 8 SSR loci were equal to those detected when both parents were known.

DISCUSSION

Theoretically, for authentic parentage identification, results based on more SSR loci with fewer alleles per locus are equal to fewer SSR loci with more alleles per locus. It is apparent that fewer SSR with more alleles per locus in parentage identification saves time, labor, and costs. In the present study, 129 alleles were detected for 4 SSR loci in the LTG PCR system. The PIC value per locus ranged from 0.765 to 0.955, with an average value of 0.8943, which demonstrated a higher level of polymorphism (PIC > 0.5). The quadruple PCR systems (Li et al. 2012) enabled efficient genotyping of the 2507 recaptured samples and separation of the subsequent PCR product.

It was obvious that the same number of SSR loci produce different exclusion probabilities and different LOD scores under conditions where both parents, a single parent, or no parent are known. Eight SSR loci produced 92.9% cumulative exclusion probability from the parentage identification based on a single parent identified in turbot *Scophthalmus maximus* L. (Yu et al. 2009). Authentic parentage identification under conditions with 1 unidentified parent requires additional SSR loci compared with conditions when both parents are known. Gu et al. (2012) assigned 647 offspring to correct full-sib families in Jian carp *Cyprinus carpio* var. Jian based on 16 SSR loci, in which >94.6% of the progeny were assigned correctly with both parents unknown. In the present study, only 4 SSR loci in the LTG system produced a ≥99.99% cumulative exclusion probability with an LOD score of ≥3.0 for the parentage identification; inclusion of the additional 4 SSR loci did not affect the results (Table 4). For parentage identification with a single parent known, 4 SSR loci in the LTG produced a 99.42% cumulative exclusion probability (Table 2); 30 and 44 parentage relationships were detected with an LOD score of 3.0 (or greater) where the paternity or maternity were known, respectively. With the inclusion of the additional 4 SSR loci for the HTG, the cumulative exclusion probability increased to >99.99%. However, the number of parentage rela-

Table 4. *Fenneropenaeus chinensis*. Logarithm (base 10) of odds (LOD) score in the double-parent and single-parent parentage testing for families A to F. Values outside and inside parentheses are the LOD score from parentage analysis based on 4 and 8 short sequence repeat (SSR) loci, respectively. Left column: detected parentage in each family

	Parentage analysis with both parents known				Parentage analysis with paternity known					Parentage analysis with maternity known				
	A	B	C	E	A	B	C	D	E	A	B	C	D	E	F
1	3.16 (3.54)	3.04 (3.63)	5.92 (5.26)	6.12 (6.41)	5.66 (5.78)	6.22 (5.89)	6.17 (6.44)	3.02 (2.23)	5.86 (5.45)	5.48 (5.23)	6.25 (6.05)	7.09 (6.88)	6.68 (2.21)	8.59 (7.56)	4.08 (2.02)
2	6.66 (6.27)	5.07 (5.71)	4.18 (4.32)		6.61 (5.38)	5.13 (5.33)	5.18 (4.88)	3.74 (2.01)		6.67 (5.88)	5.88 (6.56)	6.26 (5.77)	5.59 (1.32)	4.38 (2.66)	3.66 (0.88)
3	3.68 (3.87)				4.08 (4.17)	4.28 (0.97)	4.39 (1.23)			4.36 (4.42)	3.64 (1.56)	3.56 (1.03)	4.01 (1.76)	6.79 (2.56)	4.52 (1.67)
4					3.96 (1.07)	3.06 (1.03)	3.26 (0.67)			3.07 (0.78)		4.44 (1.73)	3.25 (1.14)		4.09 (1.11)
5					3.12 (1.77)	3.36 (1.25)	3.03 (0.98)			4.14 (1.33)		4.98 (1.31)	4.37 (2.36)		5.78 (1.48)
6					3.06 (1.38)	3.27 (0.74)	3.16 (1.34)			3.64 (1.34)		3.49 (0.96)			3.34 (1.04)
7					4.36 (1.21)	4.18 (1.68)	3.68 (2.01)			3.04 (1.72)		3.42 (2.16)			3.22 (1.55)
8					3.22 (1.63)	3.22 (1.64)	4.17 (2.01)			3.57 (0.59)		3.32 (0.89)			3.46 (1.23)
9					4.11 (2.16)	5.56 (2.02)	3.54 (0.78)			4.64 (0.98)		6.40 (2.02)			
10												4.98 (1.68)			
11												4.61 (1.43)			
12												3.75 (0.66)			
13												3.06 (1.54)			
14												5.49 (1.21)			
15												6.88 (1.31)			
16												3.57 (1.08)			

tionships detected with greater than a 3.0 LOD score decreased to 8 for the groups with a known paternity or maternity (Table 4); the observed parentage relationships were similar to the results when both parents were known. Thus, both the exclusion probability and LOD score were important in confirming an authentic parentage relationship.

Based on the parentage identification results when both parents were known, we tested and confirmed the accuracy of single-parent identification in *Fenneropenaeus chinensis* using SSR loci. Due to the diversity of mating behaviors among aquatic species, in most cases, the parents may not be available simultaneously. The lack of unclear candidate paternities is a challenge for selective breeding programs and ecological research. The present findings provide an alternate method for identification of single-parent parentage, especially in aquatic animals. The present study is a successful attempt in tracing individuals released from hatcheries.

In a previous study (Wang et al. unpubl.), we developed a recapture assessment in hatchery release by using SSR loci in individual tracing of *Fenneropenaeus chinensis*. However, the culturing of 3 full-sib families was tedious and prone to failure. Furthermore, the development of full-sib families had to be synchronized with the hatchery-released stock. Could we omit this process in a molecular marker recapture program? Due to the unique habitat, the spawners collected by the hatchery from sea every spring were all female shrimp carrying spermatophores. The present study has confirmed the accuracy of single-parent parentage identification in a full-sib family. Our SSR application to trace single parentage relationships in *F. chinensis* will simplify the aquaculture techniques for this species by skipping the culturing of full-sib families, and potentially tracing all hatchery-released individuals.

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