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Development and validation of SNP genotyping assays to identify genetic sex in the swimming crab *Portunus trituberculatus*

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ABSTRACT

The swimming crab *Portunus trituberculatus* is an economically vital aquaculture species in China and South East Asia. Monosex female culture has been identified as a key priority due to their increased market value, consumer preference and enhanced growth performance. However, the lack of a rapid and reliable sex-linked marker panel has hampered the application of sex control and understanding of the sex determination in the species. In this study, two sex-linked SNPs obtained from our simplified genomic data were validated by sequencing and then converted into competitive allele-specific PCR assays. The results showed high consistency between the genetic and phenotypic sex providing therefore a rapid test sex identification. Moreover, heterogametic genotypes in males provided a significant genetic evidence to support an XX/XY sex determination system in the *P. trituberculatus*.

1. Introduction

The swimming crab *Portunus trituberculatus* is an important marine aquaculture species widely distributed along the coast from Japan, Korea to China (Dai, 1986; Xue et al., 1997; Katsuyuki et al., 2006). This species is one of the most economically important marine crabs found in China and has been artificially cultured since the 1990s (Hao et al., 2015). Given its superior nutritional value and sensory quality preferred by consumers, especially due to the ovary in the female individuals, female crabs have an increased commercial value than males (Fan et al., 2008). In addition, a sexual growth dimorphism has been shown in *P. trituberculatus* with females growing faster than males (Wang et al., 2018). The production of female monosex stocks would therefore increase productivity and profitability of the sector.

Previous studies have suggested that *P. trituberculatus* have an XX/XY (male heterogametic) sex determination system (Lv et al., 2018). Accordingly, all-female populations could be produced by mating sex-reversed females, *i.e.*, neomales (XX), with normal females (XX) in theory as done in other aquaculture species such as *Carassius auratus*, *Cyprinus carpio*, *Ctenopharyngodon idella*, *Oncorhynchus mykiss*, and *Salmo salar* (Robert and Yoshitaka, 2002). In other crustaceans, females/males were found to be partially or fully masculinised/ feminized following manipulations such as androgenic gland (AG) implantation (*Eriocheir japonicas,* Taihung et al., 1993), AG removal (*Macrobrachium rosenbergii*, Sagi et al., 1990; Aflalo et al., 2006), AG-specific insulin-like peptides (IAGs) silencing (*M. rosenbergii,* Ventura and Sagi, 2012; Lezer et al., 2015) and AG extracts (*Eriocheir sinensis*, Liu et al., 2006) or AG cells (*M. rosenbergii,* Levy et al., 2016) injection. These studies illustrated that sex reversal in crustaceans can be induced experimentally. However, the validation of any such induction protocol and confirmation of sex reversal effects can be challenging due to the time required for progeny testing, since the identification of sex in *P. trituberculatus* seedlings can usually be done reliably after 3–4 months of pond culture based on the shape of the abdomen (Li et al., 2020). The implementation of a rapid and reliable assay for sex would fast track the process and implementation within the industry.

The molecular marker technique has been demonstrated to be an effective tool for the identification of genetic sex in a wide variety of species (Liu and Cordes, 2004; Chen et al., 2008; Ventura et al., 2011). As a third-generation molecular marker, single nucleotide polymorphism (SNP) has been widely used in genotyping. SNP data can be obtained using one of the numerous uniplex or multiplex SNP

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genotyping platforms that combine a variety of chemistries, detection methods, and reaction formats. Competitive allele-specific PCR, one of the uniplex SNP genotyping platforms, has evolved to be a global benchmark technology and is commercially available as Kompetitive Allele Specific PCR (KASP™) by LGC Biosearch Technologies. Based on allele-specific oligo extension and fluorescent resonance energy transfer (FRET) for signal generation (http://www.lgcgenomics.com), it allows high throughput analysis, low cost, high sensitivity and specificity (Semagn et al., 2014). Many studies in aquaculture have reported the application of competitive allele-specific PCR, such as Atlantic halibut (Palaiokostas et al., 2013), Atlantic salmon (Gonen et al., 2014) and tilapia (Syaifudin et al., 2019).

In the present study, competitive allele-specific PCR assays were developed and validated for two sex-linked markers (Ptr67655 and Ptr138136) obtained from our previous study (Li et al., 2020) which will provide useful tools for producing monosex female population of *P. trituberculatus*.

2. Materials and methods

2.1. Crab materials and DNA isolation

A total 124 wild swimming crabs (62 females and 62 males, average weight (66.52 ± 11.23 g) purchased from Meishan market (Ningbo China) were used for development and validation of the assays. The phenotypic sex was assessed by observing the shape of the crab's abdomen according to Xue et al. (1997). Prior to the sampling, all crabs were treated with cold shock method to minimise suffering. Crab muscle were sampled and stored in absolute ethanol. Genomic DNA was extracted using EZNA® Tissue DNA Kit (Omega, USA) according to the manufacturer's instructions. Quality and concentration of the extracted genomic DNA were examined by Nano-100 Micro-Spectrophotometer (Allsheng, China).

2.2. PCR and sequencing

Two sex-linked SNP loci (Ptr67655 and Ptr138136) determined by quantitative trait locus (QTL) mapping analysis from our previous report were amplified from 30 crabs (15 females and 15 males) using primers PS7F, PS7R and PS8F, PS8R (Table 1, Li et al., 2020). The PCR reaction was performed in a 50 μL reaction volume containing 25 μL 2×Super Pfx MasterMix (CWBiotech, China), 2 μL of genomic DNA, 2.5 μL of each primer (10 μM), 18 μL of PCR-grade water. The PCR amplification conditions were: 98 °C for 3 min followed by 35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 15 s, and then 72 °C for 10 min. The PCR products were gel-purified prior to being sequenced (ABI3730xl, USA) in both directions. The results were verified using SeqMan software (DNASTAR, USA) to confirm the authenticity of the SNP sites in the 30 individuals.

Table 1
Sequence information of the primers used in this study.

Primer	Sequence (5′-3′)	PCR objective
PS7F	TTAAGTTTGAGTATTGAGTATCCAC	Sequencing
PS7R	AATGAGAAGTATTGTAAATGATGTT	Sequencing
PtS7FAM	GAAGGTGACCAAGTTCATGCTATTTTTG TACACTACACCTCCCCC	Competitive allele-specific PCR
PtS7HEX	GAAGGTCGGAGTCAACGGATTTTTGTA CACTACACCTCCCCT	Competitive allele-specific PCR
PtS7C	GCTAGAAAGGGRTGTAGCAAACAAGTT	Competitive allele-specific PCR
PS8F	ATACCAGACAAGAGGGCTTC	Sequencing
PS8R	TCCCATATAGATATTAGTGTCATTC	Sequencing
PtS8FAM	GAAGGTGACCAAGTTCATGCTAGGCTA GTGCACTGATCCTCCA	Competitive allele-specific PCR
PtS8HEX	GAAGGTCGGAGTCAACGGATTGGCTA GTGCACTGATCCTCCG	Competitive allele-specific PCR
PtS8C	CTGTCAACTTCACCTCAAGTTGATGTTA	Competitive allele-specific PCR

2.3. Development of competitive allele-specific PCR assays

KASP™ primers including two allele-specific forward primers and one common reverse primer (Table 1) of each SNP for the assay, were designed based on the sequences selected by LGC's primer picker software (LGC, USA). Bi-allelic discrimination was achieved through the competitive binding of two allele-specific forward primers, each with a unique tail sequence that corresponded with two universal FRET cassettes, one of which was labelled with FAM™ dye and the other was labelled with HEX™ dye.

The primers were first used to test 30 sequenced individuals and later used to test a total of another 94 individuals. The competitive allele-specific PCR reactions were running on a LightCycler480 instrument (Roche, Switzerland). The reaction was performed in a 10 μL reaction volume containing 5 μL 2× Master mix (LGC, UK), 4.86 μL of genomic DNA, 0.14 μL of primer mix (FAM primer (100 μM): HEX primer (100 μM): Common primer (100 μM): Nuclease-free water = 6: 6: 15: 23). And the following cycling conditions were used: 94 °C for 15 min hot start activation; 94 °C for 20 s, 61 °C (61 °C decreasing 0.6 °C per cycle to achieve a final annealing temperature of 55 °C) for 60 sec (10 cycles); 94 °C for 20 s, 55 °C for 60 s (26 cycles); 37 °C for 30 sec (1 cycle, cooling) followed by an end-point fluorescent read. Since the KASP amplicons are usually smaller than 120 bp, no extension step is necessary in the PCR protocol. If the signature genotyping groups had not formed after the initial amplification, additional 3 amplification cycles (usually 94 °C for 20 s, 57 °C for 60 s) were applied, and the samples were read again.

3. Results

3.1. sequence analysis

The PCR products of PS7 and PS8 primers were approximately 210 bp and 240 bp, respectively. All the female crabs were heterozygous (PS7 was A/A, Fig. 1A; PS8 was T/T, Fig. 1B), while the male individuals were all homozygous (PS7 was A/G, Fig. 1A; PS8 was T/C; Fig. 1B).

3.2. Assay genotyping

The competitive allele-specific PCR assays developed from the SNPs in the Ptr67655 and Ptr138136 loci were first used to test in 30 crabs. All the results from the assays were in agreement with sequence results, successfully distinguishing between homozygous females and heterozygous males. Subsequently, the assay was performed on a total of 94 individuals including females and males. Individuals clustered near the X-axis or Y-axis are homozygous, and individuals clustered at the middle are heterozygotes (Fig. 2). The results of the genetic sex of each crab based on the assays were compared to phenotypic sex. It showed that the newly developed competitive allele-specific PCR assays exhibited a 100 % reliability in identifying the genetic sex of *P. trituberculatus*.

4. Discussion

The application of DNA marker for marker-assisted selection and breeding remains limited in *P. trituberculatus*. Given the market potential of all-female monosex culture in *P. trituberculatus*, the development of sex-linked markers has become very attractive. In previous studies, sex-specific markers of *P. trituberculatus* at the family level were identified *via* segregation distortion analysis (Lv et al., 2018) and a high-resolution melting-based assay was developed for sex genotyping with a specificity of 100 % in a wild population of *P. trituberculatus* (Li et al., 2020). In this study, two competitive allele-specific PCR assays were developed based on two sex-linked SNP loci followed by a lot of testing on wild crabs. It enriched the application of molecular markers in *P. trituberculatus*.

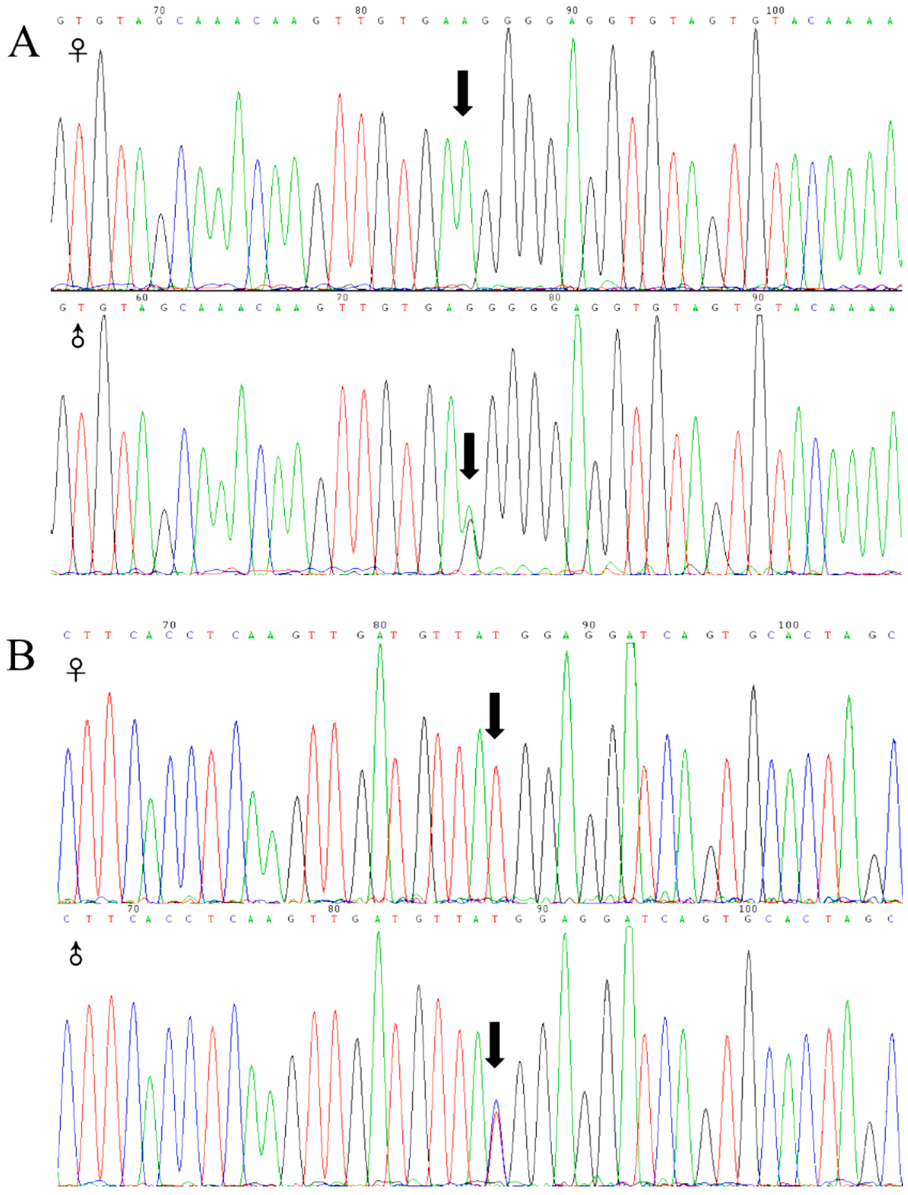

Fig. 1. Genotype of SNPs in male and female individual by sequencing. (A) PS7 and (B) PS8. (♀) female and (♂) male. The black arrows indicate the location of SNP loci.

Single nucleotide polymorphism has been widely used in genotyping. By now, there are several published methods available for SNP genotyping, such as single base extension-tag microarrays (Sobrino et al., 2004), MALDI mass spectrometry (Tost and Gut, 2002), dual-colour hybridisation (Liu et al., 2007), nanoscale engineered biomagnetite (Matsunaga et al., 2007), pyrosequencing (Hayford et al., 2011), TaqMan probe (Hansen et al., 2011), DNA-mounted self--assembly (Bichenkova et al., 2011), SNP chips (Song et al., 2012), Tm-shift assay (Feng et al., 2014) and high-resolution melting (Taylor, 2009). The competitive allele-specific PCR assay genotyping is a method of choice for SNP genotyping, because of its high throughput, low cost, high sensitivity and specificity (Semagn et al., 2014). The results from the current study showed that the two assays based on competitive allele-specific PCR exhibited a 100 % reliability in identifying the genetic sex of *P. trituberculatus*. This study provides an efficient method for fast and reliable SNP genotyping analysis of many samples in a short time.

Based on the heterogametic genotypes found in males in previous studies (Li et al., 2020), it was suggested *P. trituberculatus* has a XX/XY sex determination system. In the current study, a total 124 wild crabs were genotyped by competitive allele-specific PCR assays and results confirmed that all males genotypes analysed were heterogametic while females were homozygous. These results confirmed previous results with an XX/XY sex determination system in *P. trituberculatus*.

In conclusion, two sex-linked SNPs were successfully converted into competitive allele-specific PCR assays. The new validated assays obtained in this study will have potential applications in the elucidation of the evolution of sex chromosomes and the production of monosex populations in *P. trituberculatus*.

Author contributions

Conceptualization, R.L. and C.L.; Methodology, R.L. and M.B.; Formal analysis, J.L. and R.L.; Investigation, J.L., X.L., Q.C., W.Z. W.S. and C.M.; Writing - original draft preparation, J.L., R.L., M.B. and H.M.; Writing - review and editing, funding acquisition, R.L. and C.W.

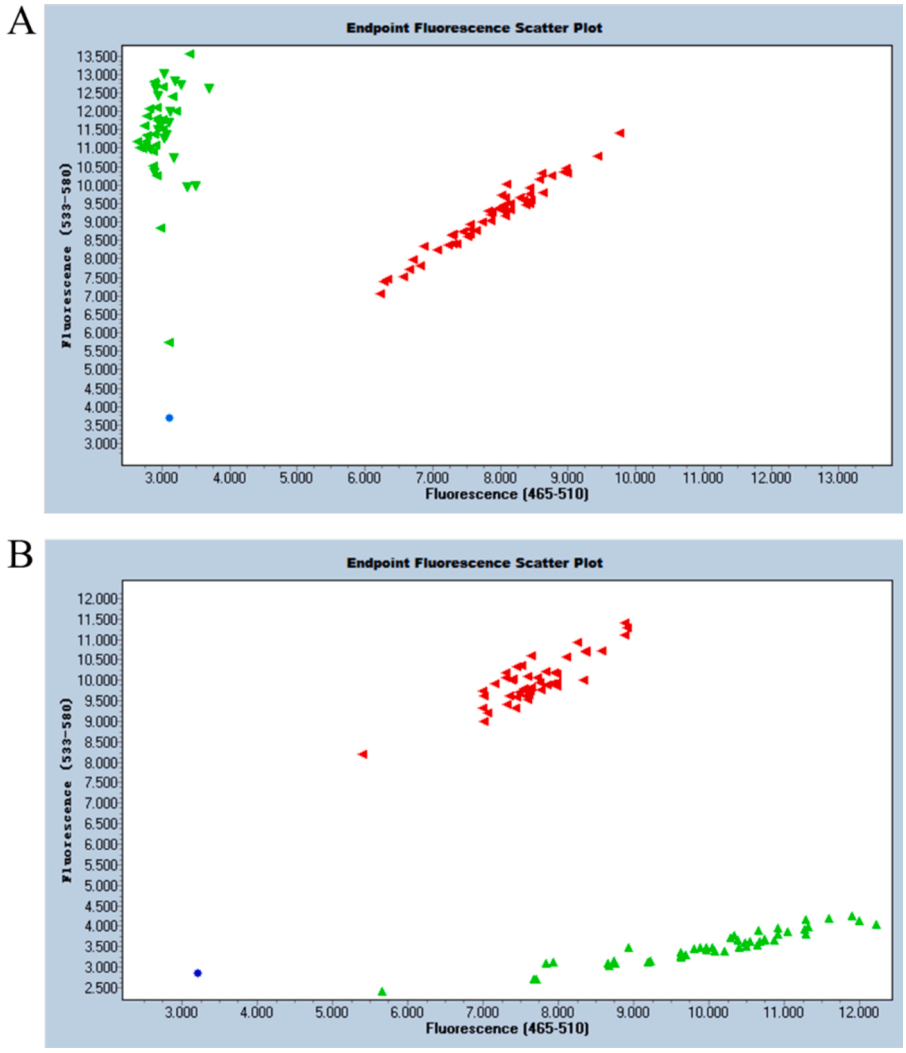

Fig. 2. Genotypic data from the competitive allele-specific PCR assays. (A) PtS7 and (B) PtS8. The X-axis indicates FAM fluorescence units, and the Y-axis indicates HEX fluorescence units. The green dots indicate females while the red dots indicate males. And the blue dots in the lower left indicate a water control (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Ethical statement

This study was approved by the Ethics Committee of Ningbo University, and conducted according to relevant national and international guidelines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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