

ORIGINAL ARTICLE

Isolation and characterization of a female-specific DNA marker in the giant freshwater prawn *Macrobrachium rosenbergii*

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In this study, a female-specific DNA marker in the freshwater prawn *Macrobrachium rosenbergii* was identified through amplified fragment length polymorphism (AFLP). The AFLP-derived sequence-characterized amplified region (SCAR) marker was tested in over 200 individuals, giving reproducible sex identification. Further molecular characterization of the sex-marker's genomic region (~3 kb long) revealed the presence of tandem and inverted repeats. The ~3-kb sequence was identified both in male and female prawns, but with subtle differences: a deletion of 3 bp (present in female prawn but absent in male prawn) identified upstream of the SCAR marker sequence and two female-specific

single-nucleotide polymorphisms, both indicating that male prawns are homozygous, whereas female prawns are heterozygous in this locus. Fluorescent *in situ* hybridization showed the ~3-kb sequence to be unique: to the best of our knowledge, this is the first report of a unique sex-specific sequence observed *in situ* in crustaceans. The sex-specific marker identified in *M. rosenbergii* may have considerable applied merit for crustacean culture in that it will enable the determination of genetic sex at early developmental stages when phenotypic differences are not identifiable. *Heredity* (2011) **107**, 456–461; doi:10.1038/hdy.2011.32; published online 27 April 2011

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Introduction

The development of molecular methods has led to the identification of molecular markers in a wide variety of species (Liu and Cordes, 2004; Granevitze *et al.*, 2007). As a result, molecular markers and associated tools, such as genetic maps, are currently available for some crustacean species, particularly for shrimps. Molecular markers have been applied, for example, for analyzing the genetic diversity of wild shrimp resources (Xu *et al.*, 2001), for strain and species identification in penaeids (Calo-Mata *et al.*, 2009), and for parentage analysis in the black tiger shrimp *Penaeus monodon* (Jerry *et al.*, 2006). In addition, linkage maps have been generated for several penaeid shrimp species, in most cases by using amplified fragment length polymorphism (AFLP) (Li *et al.*, 2003; Zhang *et al.*, 2007; Staelens *et al.*, 2008). For prawns, however, no sex-specific markers have been identified, and to date *P. monodon* is the only decapod crustacean species for which sex-linked markers have enabled PCR-based differentiation of male from female prawns (Staelens *et al.*, 2008).

Only very few invertebrate species are known to carry WZ sex chromosomes (Fuková *et al.*, 2007), although

cumulative evidence suggests that WZ chromosomes are common in macruran decapod crustacean species (crayfish, lobsters, spiny lobsters, shrimps and prawns) (Staelens *et al.*, 2008). Evidence for type and/or presence of sex chromosomes is drawn from physical mapping of sex-specific sequences, from sex-linked linkage maps, from karyological studies and from crosses of sex-reversed individuals (Legrand *et al.*, 1987; Sagi and Cohen, 1990; Griffiths and Orr, 1999; Li *et al.*, 2003; Parnes *et al.*, 2003). In Crustacea of the order Decapoda, karyotype studies have revealed distinguishable sex chromosomes only in crabs (Brachyura) and hermit crabs (Anomura), as reviewed by Parnes *et al.* (2003). In all nine species reviewed, the male prawn was found to be the heterogametic sex, bearing XY chromosomes. In the few macruran species for which karyotype studies are available, including those in the giant freshwater prawn *Macrobrachium rosenbergii* (Justo *et al.*, 1991), sex chromosomes are morphologically indistinguishable from autosomes. It is believed that in *M. rosenbergii*, as in other macruran species, the female prawn is the heterogametic sex, bearing WZ sex chromosomes, whereas the male prawn is the homogametic sex, bearing ZZ sex chromosomes. Data supporting this premise may be taken from two sex reversal studies: In one study, mating of sex-reversed male prawns with normal male prawns produced all-male (ZZ × ZZ) progeny (Sagi and Cohen, 1990; Aflalo *et al.*, 2006). In the other study, the progeny of sex-reversed female prawns mated with normal female prawns (WZ × WZ) exhibited a sex ratio skewed to female prawns (Malecha *et al.*, 1992).

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In this study, we aimed at identifying sex-specific markers in *M. rosenbergii* by using AFLP. We isolated the first sex-specific genomic marker for this species and successfully converted it into a female-specific sequence-characterized amplified region (SCAR) marker, thereby enabling PCR-based sex identification based on several female-specific polymorphic features. Fluorescent *in situ* hybridization (FISH) provided proof that the genomic region in the vicinity of the sex-marker constitutes a unique sequence, as it appears only in one locus in the genome. On the basis of our findings, we posit that the sex marker is probably located in the sex chromosome, in a locus common to the Z and W chromosomes.

Materials and methods

Animals

The broodstock for the *M. rosenbergii* prawns currently being cultured in Israeli aquafarms was imported into Israel from Hawaii approximately two decades ago. The prawns used for this study were maintained at Ben-Gurion University of the Negev under the following conditions: Food comprising shrimp pellets (Rangen Inc., Buhl, ID, USA; 30% protein) was supplied *ad libitum* three times a week. Water quality was assured by circulating the entire volume through a bio-filter maintaining all the water physicochemical parameters as described before (Khalaila *et al.*, 2002). Each egg-berried female prawn was transferred to a separate closed 100-L tank containing 12–16 ppt salt water, which was circulated through a 100- μ m mesh net. After the brood had hatched, the female prawn was removed from the tank, and the newly hatched larvae were maintained according to the protocol devised by Uno and Chin Soo (1969) and fed with *Artemia* nauplii daily. Prawns were also sampled from two other populations, one grown by Ms Ayana Perlberg of the Dor Research Center and the other by Dr Shmuel Parnes of the ornamental aquaculture company, Colors (Moshav Hatzeva, Israel).

Genomic DNA extraction

Genomic DNA was extracted from frozen tail muscle tissue with a Genra Puregene Tissue Kit (Qiagen, Chatsworth, CA, USA) or REDExtract-N-Amp Tissue PCR Kit (Sigma, St Louis, MI, USA), according to the manufacturer's instructions.

AFLP reaction

AFLP was carried out according to the method of Kashkush *et al.* (2001). Briefly, 0.5 μ g of genomic DNA from three male and three female individuals was digested with *EcoRI* and *MseI* and ligated to adaptors. The digestion/ligation reaction was carried out at 37 °C for 2 h. The pre-selective PCR was carried out with primers complementary to the core adaptor primer, and selective amplification was carried out with [γ -³²P]ATP end-labeled *EcoRI* primer and other primers complementary to the *MseI* adaptor sequence, with three additional random nucleotides to each primer. The PCR products were mixed with 20 μ l of formamide dye (98% formamide, 10 mM EDTA, 0.1% (w v⁻¹) bromophenol blue, 0.1% (w v⁻¹) xylene cyanol), heated at 95 °C for 5 min and separated on 5% denaturing polyacrylamide standard sequencing gel (40% acrylamide:bisacrylamide

at a 1:20 ratio, 7.5 M urea, 1 \times Tris borate EDTA-TBE buffer, 43 cm long) at 55 W for 1.5 h (after 40 min of pre-run under the same conditions). Gels were then dried on a gel drier for 2 h at 70 °C and exposed to X-ray film (Fuji, Tokyo, Japan) for about 16 h at room temperature. Unique male and female bands were extracted from the gel, re-amplified under the same PCR conditions as used for selective amplification, cloned and sequenced.

Sequence-characterized amplified region

Specific primers were designed to amplify each of the isolated AFLP bands. PCR was performed with 100 ng of genomic DNA, 1 μ M of forward primer and 1 μ M of reverse primer (primer sequences are available upon request), 12.5 μ l of Ready Mix REDTaq (Sigma, St Louis, MO, USA) and water to a final volume of 25 μ l. PCR conditions were: 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C, followed by 35 cycles. PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light with ethidium bromide.

Genome walking

Genome walking was carried out using DNA Walking SpeedUp Premix Kit (Seegene, Rockville, MD, USA), according to the manufacturer's specifications.

Fluorescent *in situ* hybridization

FISH was carried out as described by Dzikowski *et al.* (2007), with the exceptions that the prawn sample was prepared according to Justo *et al.* (1991) and that the probe used was the PCR probe of nucleotides 1–2003 of the ~3 kbp fragment identified (primers available upon request). Samples were prepared from several male and female gonads, and the signal was scored for a total of 74 nuclei that could be distinguished morphologically. On the basis of the nucleus morphology, the signals were scored positively for one dot (haploid cells) or two dots (diploid cells). In some cases, two double-dotted spots were also scored.

Sequence analysis

Sequence annotation relied on the BLAST 2.0 package from NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) and from the Institute for Genomic Research (<http://tigrblast.tigr.org/tgi/>). Repetitive sequences were identified by the self-blast tool (<http://www.proweb.org/proweb/Tools/selfblast.html>) and the tandem repeats finder (<http://tandem.bu.edu/trf/trf.html>) (Benson, 1999). Open reading frames along the sequence were viewed with the NEBcutter (<http://tools.neb.com/NEBcutter2/>) (Vincze *et al.*, 2003). Indel identification in uncloned sequences was obtained by Shift Detector (<http://cowry.agri.huji.ac.il/cgi-bin/frshplus.cgi>) (Seroussi *et al.*, 2002).

Results and discussion

Sex-specific AFLP markers

The AFLP assay is an ideal system for the identification of sex-specific DNA markers, because it requires only a small amount of genomic DNA for the study of hundreds of genomic loci in a relatively short time and, more importantly, in an unbiased manner (Vos *et al.*, 1995). In addition, AFLP is ideal for studying poorly deciphered

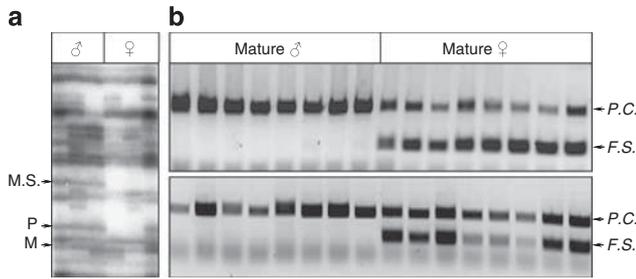


Figure 1 Amplified fragment length polymorphism (AFLP) screening for sex-specific genomic sequences in *M. rosenbergii* and PCR of genomic DNA extracted from mature *M. rosenbergii*. (a) AFLP profiles of three male and three female mature *M. rosenbergii* prawns obtained with a combination of *Eco*-NN- and *Mse*-NN-selective primers ('N' represents a randomly picked nucleotide). Representative monomorphic (M), polymorphic (P) and male-specific (MS) sequences are indicated by arrows. (b) The female-specific genomic sequence (FS) is amplified in all mature female specimens and is absent in all mature male specimens, as shown by PCR. Positive control (PC), which was derived from an AFLP monomorphic band, is present in all specimens. Primer sequences are available upon request.

genomes, such as the genome of the freshwater prawn *M. rosenbergii*, because it does not require any previous knowledge about the genomes. Using 18 primer combinations in the AFLP reaction, we screened six prawns—three males and three females—with the aim to identify sex-specific markers. A representative AFLP product is shown in Figure 1a. Of a total of 1795 clearly distinguishable AFLP bands screened, 65 were specific to male prawns, and 87 specific to female prawns. The sex-specific bands could be classified into four groups: (1) 12 bands were present in all three male prawns and absent in all three female prawns; (2) 16 bands were present in all three female prawns, but absent in all three prawns; (3) 54 bands were present in one or two male prawns and absent in all three prawns; and (4) 71 bands were present in one or two female prawns, but absent in all three prawns. These data suggested that the bands classified into groups 1 and 2 could be sex specific and hence could serve as sex-specific markers.

Validation of the sex-specific bands by site-specific PCR
To convert AFLP markers into SCAR markers, the 16 female-specific and the 12 male-specific bands identified above were extracted from the gel and sequenced. Specific primers were designed for each sequence and used in the PCR amplification with DNA from four male prawns and four female prawns as the template. In 27 of the cases, monomorphic PCR bands were observed in both male and female cases, thereby making it difficult to convert these bands into SCAR markers. In one case, however, a PCR product (~140 bp long) was amplified for the female prawns, but was absent for the male prawns (Figure 1b; FS). This SCAR marker was tested in over 200 individuals drawn at random from the three populations of prawns described above (see Materials and methods), with 100% success in the identification of female prawns (see examples in Figure 1b and Supplementary Figure S1). Our success rate (1 out of 28) for converting an AFLP marker into a SCAR marker falls in the range of the success rates reported in previous studies in salmonids—one out of 15 reported by Felip *et al.* (2005) and one out of 52 reported by Brunelli and

Thorgaard (2004). We posit that it might be possible to convert some of the other 27 sex-specific AFLP bands into SCAR markers by using genome walking to reveal the sequences spanning the regions outside the AFLP marker, as was carried out for the male-sterility marker in the plant *Brassica napus* (Zeng *et al.*, 2009) and in potato; in the latter study, genome walking facilitated the successful conversion of six of 10 AFLP markers to SCAR markers (Brugmans *et al.*, 2003). Genome walking was necessary in the above six instances owing to the fact that the single-nucleotide polymorphisms (SNPs) resided in the restriction site. In our study, the finding of a female-specific sex marker—rather than a male-specific marker—was not surprising, as it is believed that in *M. rosenbergii* sex determination is governed by a WZ/ZZ mechanism (Sagi and Cohen, 1990).

Molecular characterization of the sex-specific marker and its genomic vicinity

DNA sequencing of genomic regions flanking the SCAR marker (~3 kb) in female prawns revealed the presence of seven tandem repeats, each of 88 bp, residing 83–698 bp upstream of the sex-specific marker. Each tandem repeat started with a 12-bp palindrome sequence (Figure 2a). In addition, two 11-bp inverted repeats were identified further upstream (starting at 432 and 794 bp after the tandem repeats), and yet another one 614 bp downstream to the sex-specific marker (Figure 2a). Another set of primers (Figure 2b, P1 and P2) flanking the SCAR marker was used for amplifying a larger DNA fragment of ~340 bp (Figure 2b). Surprisingly, PCR amplification of the 340-bp fragment produced positive products for both male and female prawns. Sequence analysis of this fragment revealed that the original AFLP sex-marker included two female-specific SNPs in the primer location (P3 in Figure 2b), which enabled the production of the SCAR female-specific marker (Figure 1b). Moreover, three nucleotides located between the SCAR sequence and the tandem repeats sequence, within the 340-bp amplified fragment, were present in all the cloned male sequences, but absent in some of the female clones (Figure 2b, ∇). PCR amplification using a forward primer designed on the basis of female-specific deletion (P4 in Figure 2b) and a reverse primer downstream of the SCAR sequence (P2 in Figure 2b) produced positive products only in female prawns (Figure 3a, top panel). However, PCR amplification using a forward primer that included the three nucleotides (P5 in Figure 2b) and a reverse primer downstream of the SCAR sequence (P2 in Figure 2b) produced positive products for both male and female prawns (Figure 3a, bottom panel). Un-cloned sequences amplified from DNA of five female and five male prawns by using primers P2 and P5 were obtained. For all five male prawns, the sequences resulted in single peaks (Figure 3b, left bottom), whereas for all five female individuals, the sequences contained overlapping peaks (Figure 3b, left top). The female sequences were treated with Shift Detector software (Seroussi *et al.*, 2002), where the 3-bp deletion in female prawns was detected with a probability score of 1.53×10^{-5} . In addition, sequencing of un-cloned female-specific PCR products obtained in the previous PCR (using primers P4 and P2; Figure 2b) produced single peaks, whereas no amplification was found for male prawns (Figure 3b right).

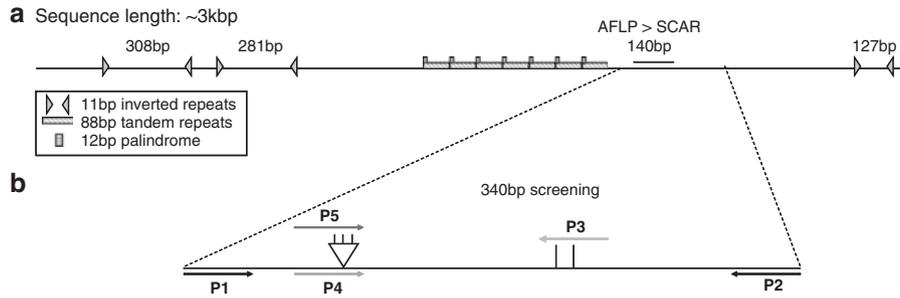


Figure 2 Schematic overview of the original sex-specific sequence found by amplified fragment length polymorphism (AFLP) and the further upstream and downstream sequence features obtained by genome walking and sequence characterization. (a) The ~3-kbp sequence obtained by AFLP and genome walking. Seven tandem repeats of 88 bp, each starting with a 12-bp palindrome, are indicated by striped rectangles, as are three 11-bp inverted repeats (indicated by triangles). (b) A 340-bp sequence within the ~3-kbp sequence obtained by genomic walking in the area surrounding the original AFLP product was amplified both in male and female prawns. In this 340-bp sequence, two female-specific SNPs were identified (), which enabled the female-specific amplification of the SCAR marker. An insertion of three nucleotides () was identified in all male sequences, but only in some of the female ones. Primers that include () or bypass () the insertion were designed. A full color version of this figure is available at the *Heredity* journal online.

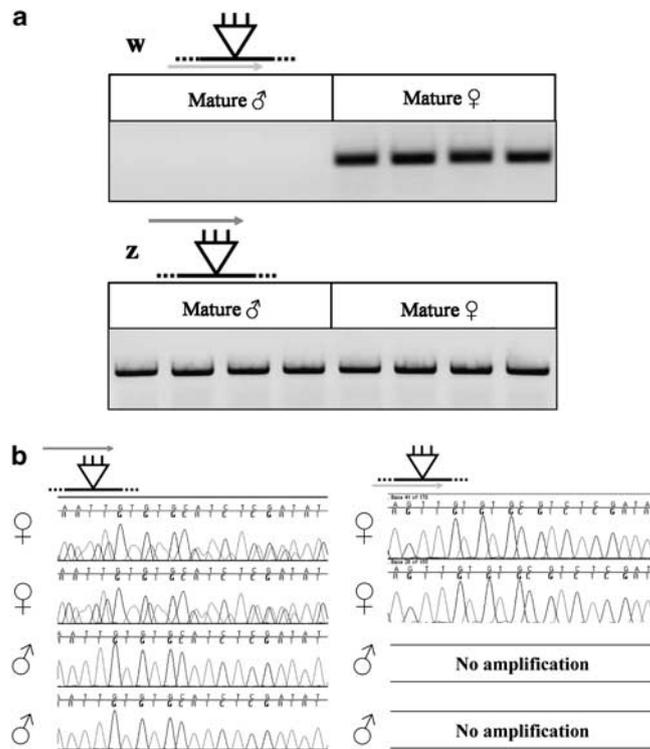


Figure 3 Gender dimorphic genomic patterns in the region of the original amplified fragment length polymorphism (AFLP) product. The 3-bp insertion found in the proximity of the AFLP marker was further analyzed by using primers that bypass or include the insertion against a common reverse primer (P2 in Figure 2a). (a) The primer that bypasses the insertion () amplified a fragment only in female prawns (top), but the primer that includes the insertion () amplified a fragment both in male and female prawns (bottom), leading to the hypothesis that this genomic sequence is homozygous in male prawns (ZZ) and heterozygous in female prawns (WZ). The primer that bypasses the insertion amplified only the W fragment, which is unique to female prawns, and the primer that includes the insertion amplified the Z fragment common to both male and female prawns. (b) When the primer that includes the insertion was used, un-cloned sequences amplified from both male and female prawns showed that the male sequence produced single peaks and the female sequence included overlapping peaks; this finding thus confirmed that female prawns are heterozygous in this region. When the primer that bypasses the insertion was used, the female sequences produced single peaks, and there was no amplification in the male prawns. Primer sequences are available upon request. A full color version of this figure is available at the *Heredity* journal online.

In summary, we used several methodologies, all of which provided support for the conclusion that female prawns are heterozygous and male prawns are homozygous in the locus of the sex marker with subtle differences, such as a 3-bp indel and two SNPs between the two female alleles. The most solid proof for the above

notion derives from the results of our cloning experiments: we cloned and sequenced both the female-specific allele and the female/male shared allele. To confirm the indel identified in the female cloned sequences, un-cloned female sequences were investigated with Shift Detector software (Seroussi *et al.*, 2002), which

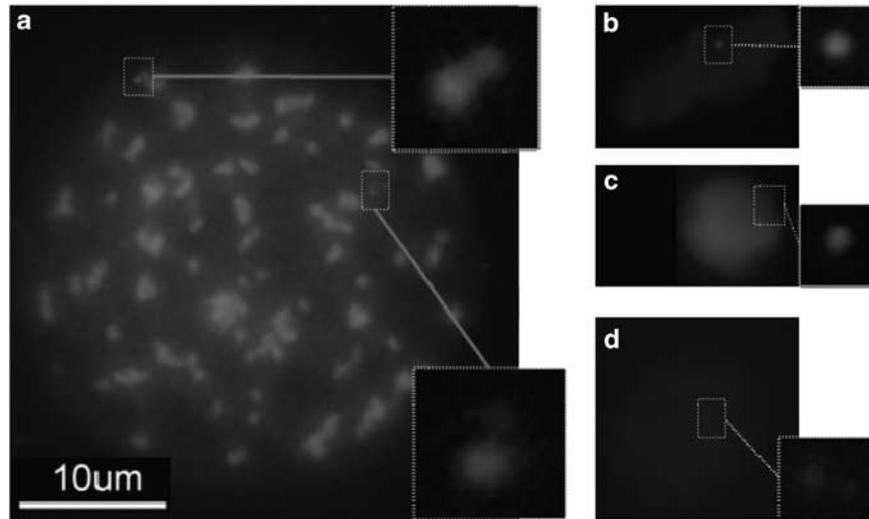


Figure 4 Fluorescent *in situ* hybridization of the sex-specific marker. Representative hybridizations from several cell types are shown. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue), and red indicates the specific signal: (a) Two double-dotted spots in a nucleus in first meiotic division (2n4c). (b) A single dot (1n1c) of a spermatozoa and a spermatid (c). (d) Two-dotted spot (1n2c) in a secondary spermatocyte (bar = 10 µm). A full color version of this figure is available at the *Heredity* journal online.

successfully identified the indel as present in the cloned sequences.

The identification of new alleles based on sequence-specific design of primers for PCR has been reported previously (Wang *et al.*, 1995; Chung *et al.*, 2003). It is worth noting that in the tiger shrimp *P. monodon*, the sex-linked SCAR marker, which resides on the sex chromosome, is also based on a short indel in the female-specific allele (Staelens *et al.*, 2008). To assess the copy number of the region, FISH was carried out on cells extracted from several male gonads; 2 kb out of the ~3 kb first identified in female prawns was used as a probe. The high similarity between the female-specific allele and the female/male shared allele enabled us to perform FISH for the male prawns, for which there is a known protocol for cell extraction (Justo *et al.*, 1991). In all cases in which the field was clean of background signals, the nuclei were scored according to their morphology and the number of signal spots. In four cases, there were two double-dotted spots in cells in their first meiotic division (2n4c; Figure 4a). Of the 47 instances of single dots (1n1c), 24 were spermatozoa (Figure 4b) and 23 were spermatids (Figure 4c). Twenty-three double-dotted spots (1n2c) were found in secondary spermatocytes (Figure 4d). The above results taken together indicate that the genomic region of the sex-specific marker constitutes a unique sequence in *M. rosenbergii*. The short unique marker sequence of ~3 kb obtained in this study should be carefully investigated in other populations with the ultimate aim of devising a global sex-specific marker for *M. rosenbergii* that will enable the sex identification of individuals from unrelated populations, as was carried out for a DNA sex marker identified in the three-spined stickleback (Griffiths *et al.*, 2000). In the basket willow (*Salix viminalis*), on the other hand, researchers identified sex-linked DNA markers derived from unrelated families (and therefore genetically variable), and thus concluded that these sex-linked markers represent a wide natural population rather than a limited experimental group (Semerikov *et al.*, 2003).

Significance of identifying sex-specific markers in *M. rosenbergii*

The significance of sex identification in *M. rosenbergii* lies in the importance of this species in aquaculture. As male prawns of the species grow faster than female prawns, it is not uncommon to find farms that hand segregate the male prawns for marketing and discard the female prawns, a method proven to increase yield and income for the growers by ~60% (Nair *et al.*, 2006). The bimodal growth pattern of *M. rosenbergii*, that is, male prawns growing faster than female prawns (Sagi *et al.*, 1986), has led to the elaboration of a biotechnology for monosex population production (Aflalo *et al.*, 2006). This biotechnology relies on a fully functional sex reversal of normal male prawns into neo-female prawns to produce all-male progeny. In the implementation of such a biotechnology, the availability of sex-specific markers could shorten the time needed for progeny testing, which is a crucial, time-consuming and labor-intensive stage in the proposed biotechnology. The nature of the genomic region revealed in this study (a unique sequence, dimorphic in female prawns and monomorphic in male prawns) has led us to hypothesize that this region resides in the sex chromosome in a locus common to the W and Z chromosomes. This idea is in keeping with the heterogametic female theory of Katakura (1989). However, as the sex chromosome of *M. rosenbergii* is not distinguishable morphologically (Justo *et al.*, 1991), further research is needed to verify this notion.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on Heredity website (<http://www.nature.com/hdy>)