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Uncovering a sex determination region and female-specific markers in the Crown-of-thorns starfish

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Abstract

Population outbreaks of Crown-of-thorns starfish (COTS) in the Indo-Pacific region have inflicted devastating damage on coral reef ecosystems. Manual culling remains the most effective control method against this marine pest, but it entails substantial labor and economic costs. Emerging research focused on the COTS biology, including molecular biology, has provided foundational insights that may support the development of new approaches to mitigate future population outbreaks. However, limited understanding of its sex determination mechanism and the absence of non-invasive sex identification methods have impeded sex-related research and the development of effective biocontrol strategies. Therefore, we performed high-coverage whole-genome re-sequencing of 30 COTS females and 30 COTS males to identify candidate sex determination regions, and developed sex-specific molecular markers. The distribution of sex-associated SNPs and sex-specific SNPs, along with the selection signatures of fixation index indicated that a 11–14 Mb physical region within COTS chromosome 10 was a candidate sex determination region. Within this region, the PKNOX2 gene, harboring numerous sex-associated SNPs and showing testis-biased expression, might influence sexual dimorphism. We successfully developed two pairs of female-specific molecular markers, which were validated in separate populations with 100% accuracy, enabling the non-invasive identification of the genetic sex in COTS. In addition, the identification of female-specific sequences and the genotypes of sex-specific SNPs suggested COTS might possess a ZZ/ZW sex determination system. Our study not only provides novel insights into the diverse sex determination mechanisms in echinoderms, but also establishes a genetic foundation for sex-related physiological research and the future development of biocontrol strategies against COTS.

Introduction

Sexual reproduction in eukaryotic organisms is a fundamental biological process that introduces novel genetic combinations, thus enhancing beneficial genetic diversity and the adaptive potential in populations (Barton and Charlesworth,1998; Otto, 2009; Ramos and Antunes, 2022). Crucially, the establishment of sexual reproduction inherently necessitates sex determination, a process with profound implications for the ecology and evolution of species. The mechanisms of sex determination vary widely among species, spanning genetic, environmental, and interactive cues that ultimately direct the development of distinct male and female phenotypes (Gamble and Zarkower, 2012; Bachtrog et al., 2014; Pokorná and Kratochvíl, 2014). To date, this diversity has been well exemplified in marine teleost fishes, which exhibit various sex determination strategies, ranging from genetic sex determination (GSD; e.g., XX/XY and ZW/ZZ) to environmental sex determination (ESD; e.g., temperature and dissolved oxygen) (Devlin and Nagahama, 2002; Li et al., 2022). In contrast, while the sex determination mechanisms in marine invertebrates are similarly diverse and complex, they remain far less understood (Picard et al., 2021).

Sex-specific molecular markers are crucial for elucidating sex determination mechanisms, especially in species where sexes are not morphologically distinguishable (Devlin and Nagahama, 2002; Saunders et al., 2024). Driven by advances in next-generation sequencing (NGS) technologies, the development of

sex-specific molecular markers based on genomic or re-sequencing data has become increasingly efficient and widespread in recent years (Goodwin et al., 2016; Wu et al., 2022; Fu et al., 2024; Zeng et al., 2024; Wang et al., 2025a). Consequently, this trend has gradually displaced traditional marker development techniques such as amplified fragment length polymorphism, simple sequence repeat and randomly amplified polymorphic DNA.

Echinoderms, a major clade of deuterostome invertebrates occupying a distinct phylogenetic position, exhibit diverse reproductive strategies, including parthenogenesis and asexual multiplication (Hart, 2002; Reich et al., 2015; Picard et al., 2021). As the closest sister taxa to chordates, echinoderms are considered promising model systems for investigating sex determination mechanisms and sexual plasticity. Preliminary investigations into sex determination mechanisms have been conducted across major echinoderm classes, including Holothuroidea (Wang et al., 2022a; Jiang et al., 2024), Echinoidea (Wang et al., 2022b; Wang et al., 2025b), and Asteroidea (Wang et al., 2025a), highlighting the considerable complexity of this process in this group. In these studies, genome-wide association studies (GWAS) based on whole-genome re-sequencing data from both sexes have emerged as a key approach for identifying candidate sex determination regions and genes. However, to date, no master sex determination gene has been conclusively identified in echinoderms (Wang et al., 2022a; Wang et al., 2022b; Jiang et al., 2024; Wang et al., 2025a), suggesting that sex determination in this group likely operates through a complex multi-gene regulatory network. Additionally, several sex-specific molecular markers have been successfully developed in echinoderms, including the sea cucumbers *Apostichopus japonicus* (Wei et al., 2021; Jiang et al., 2024; Liu et al., 2024), *Stichopus monotuberculatus* (Wu et al., 2022), and *Holothuria scabra* (E et al., 2023), the sea urchin *Mesocentrotus nudus* (Cui et al., 2021), as well as the starfish *Asterias amurensis* (Wang et al., 2025a). When coupled with polymerase chain reaction (PCR) amplification, these markers enable rapid, accurate and non-invasive identification of genetic sex in study organisms.

The Crown-of-thorns starfish (COTS; *Acanthaster* spp.) is a voracious coral predator whose population outbreaks have driven substantial coral losses and critically reduced the integrity and resilience of coral reef ecosystems, increasing their susceptibility to subsequent environmental stress (Branham et al., 1971; Yamaguchi, 1986; Vogler et al., 2008; De'ath et al., 2012; Baird et al., 2013; Hall et al., 2017; Deaker and Byrne, 2022). Moreover, the outbreak frequency of COTS populations has increased significantly across the Indo-Pacific region, thereby raising serious concerns regarding the health of critical reef ecosystems (Brodie et al., 2005; Fabricius et al., 2010; Pratchett et al., 2017).

Understanding the sex determination mechanisms in pest species can inform and enable biocontrol strategies by allowing targeted manipulation of sex ratios, fertility, and inheritance (Siddall et al., 2022; Scott and Tu, 2026). In addition, developing effective sex-specific molecular markers is a prerequisite for the identification of sex attractants, which could then be used to induce aggregation of pests during the breeding season for targeted removal. As a broadcast spawner with high seasonal fecundity (Babcock et al., 2016; Pratchett et al., 2021a; Uthicke et al., 2024), COTS relies on synchronized spawning events triggered by pre-spawning aggregations. The scale and synchrony of subsequent gamete release directly

influence reproductive success, which may initiate population outbreaks (Babcock and Mundy, 1992; Caballes and Pratchett, 2017; Caballes et al., 2021; Deaker and Byrne, 2022). Previous studies have supported the presence of secreted factors (pheromones) in male seminal fluid that are capable of attracting and inducing spawning in other COTS individuals (Beach et al., 1975; Hall et al., 2017; Jönsson et al., 2022). Correspondingly, transcriptomic analyses have revealed differential expression of putative chemosensory receptor genes between females and males (especially in sensory tentacles), which may function in detecting such chemical signals (Roberts et al., 2018; Jönsson et al., 2022; Morin et al., 2024). Therefore, deciphering the signals and chemosensory mechanisms underlying pre-spawning aggregation in COTS may provide a potential strategy to disrupt these events and thereby control subsequent population outbreaks (Motti et al., 2017; Harris et al., 2025).

In this study, we performed high-coverage whole-genome re-sequencing of 30 female and 30 male COTS individuals, aiming to identify a sex determination region and develop sex-specific molecular markers. The findings are expected to offer novel insights into echinoderm sex determination and a foundation for the biocontrol of COTS population outbreaks.

Materials and methods

Sample collection

In April 2024, 159 adult COTS individuals were collected from Langhua Reef (16°58'N, 112°33'E) in the Xisha Islands. Live specimens were subsequently transported to the Hainan Academy of Ocean and Fisheries Sciences aboard fishing vessels fitted with flow-through live wells. The sex of sampled individuals was determined through microscopic examination of gonad tissue. Following sex identification, gonad tissues were dissected from 30 females and 30 males, then briefly washed with 1× phosphate-buffered saline (PBS). Then, the collected tissues were flash-frozen in liquid nitrogen, and stored at -80°C. In August 2025, we additionally collected 11 female and 12 male COTS individuals from the coastal waters of Sunny Bay (18°19'N, 109°63'E), and sampled their tube foot tissues for marker validation.

DNA extraction and whole-genome re-sequencing

Genomic DNA (gDNA) was extracted from gonad and tube foot tissues using the TIANamp Marine Animals DNA Kit (TIANGEN, China) according to the manufacturer’s protocols. The concentration and quality of gDNA samples were detected through Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis.

High quality gDNA samples isolated from gonad tissues were fragmented into an average size of approximately 350 bp through sonication. Subsequently, whole-genome re-sequencing libraries were constructed as follows: end repair, A-tailing, adapter ligation, purification, and PCR amplification (Huang et al., 2017). Following standard procedures, a total of 60 libraries were sequenced on DNBSEQ-T5

platform (BGI, China) to generate paired-end 150bp (PE150) reads. These libraries were sequenced to an average depth of approximately 43.51× (ranging from 36.51× to 53.26×).

SNP calling and quality control

Adapters and low-quality reads were removed from raw reads using fastp (v0.24.0) (Chen, 2023) with default parameters. The obtained clean reads were mapped to a COTS chromosome-level genome (Liu et al., 2025) using BWA-MEM2 (v2.2.1) (Vasimuddin et al., 2019). Subsequently, SAMtools (v1.18) (Li et al., 2009) was used to sort alignment files by genomic coordinate and mark PCR duplicates. SNP calling was performed using the Genome Analysis ToolKit (GATK, v4.1.9.0) (McKenna et al., 2010), employing HaplotypeCaller for per-sample variant calling, CombineGVCFs for merging outputs, GenotypeGVCFs for joint genotyping, and SelectVariants for SNP extraction.

SNPs were filtered using GATK's VariantFiltration (parameters: 'QD < 2.0 || MQ < 40.0 || FS > 20.0 || SOR > 3.0 || MQRankSum < -2.0 || MQRankSum > 4.0 || ReadPosRankSum < -3.0 || ReadPosRankSum > 3.0'). Further filtering was performed using VCFtools (v0.1.16) (Danecek et al., 2011) with the parameters '--maf 0.1 --min-meanDP 15 --max-meanDP 100 --minGQ 30 --max-missing 0.95 --min-alleles 2 --max-alleles 2'.

Identification of candidate sex-determining region

A GWAS was conducted to identify sex-associated SNPs using plink (v1.90b6.21) (Purcell et al., 2007), with sex treated as a binary trait coded as 1 (female) and 2 (male). Manhattan and QQ plots were visualized using CMplot (v3.6.2) (Yin et al., 2021) in R. In the present study, we defined two significance thresholds ($P < 1e-5$ and $P < 1e-8$) to identify genomic regions with strong association signals.

For each SNP, fixation index F_{ST} was calculated using VCFtools (v0.1.16) to quantify the allele frequency differences between sexes. We mainly examined the SNPs exhibiting $F_{ST} > 0.25/0.5$, which are indicative of strong selective signatures. In addition, the sex-specific SNPs were detected using the Perl script (Li et al., 2020) with the parameter '-c 30 -e 0', representing the SNPs that are consistently heterozygous in one sex and homozygous in the corresponding sex. To locate the chromosome exhibiting the most pronounced differentiation between sexes, principal component analysis (PCA) was performed for SNPs on each chromosome and genome-wide SNPs.

To investigate whether a syntenic sex determination region exists in Asteroidea, we performed synteny comparison between the 21 chromosomes of COTS and the 22 chromosomes of *A. amurensis* using jcvi (v1.3.8) (Tang et al., 2024).

Exploration of sex-determination-related genes

The sex-associated SNPs (*P*-value < 1e-8) were annotated using SnpEff (v4.3t) (Cingolani et al., 2011) to identify potential sex-determination-related genes. To examine the expression patterns of these genes between testes and ovaries, we analyzed available gonad transcriptome data from both sexes (SRR18531332-SRR18531337) (Jönsson et al., 2022). In brief, the sequencing reads were aligned to the reference genome using Bowtie2 (v2.5.4) (Langmead and Salzberg, 2012), transcript abundance was quantified using HTSeq (v2.1.2) (Anders et al., 2015), and differential expression analysis was subsequently performed with DESeq2 (v1.20.0) (Love et al., 2014).

Screening of sex-specific markers

The workflow for developing sex-specific molecular markers was summarized in Fig. S1. Female (NCBI accession number: GCA_030586425.1) and male (NCBI accession number: GCA_030586445.1) scaffold-level reference genomes of COTS were downloaded from NCBI for sex-specific sequence identification. The re-sequencing reads from 5 female (F01-F05) and 5 male (M01-M05) COTS individuals were aligned to the two reference genomes using BWA (v0.7.17-r1188) (Li and Durbin, 2010). Properly aligned paired-end reads were extracted with SAMtools (v1.18) for the preliminary screening of putative female/male-specific sequences. To eliminate false-positive sequences, the secondary screening was conducted using the pooled re-sequencing data from all female or all male COTS individuals. For the candidate female-specific sequences identified, coverage information was visualized using Integrative Genomics Viewer (IGV, v2.17.3) (Robinson et al., 2023), and corresponding sex-identification primers were designed using Primer3Plus (v3.3.0) (Untergasser et al., 2012).

Validation of sex-specific molecular markers

To validate the candidate sex-specific primers, gDNA of gonad tissues from 10 re-sequenced females and 10 re-sequenced males were subjected to PCR amplification for initial screening. To control for potential amplification failures, a second primer pair targeting the 18S rDNA sequence was employed as a positive control. PCR reaction mixture (20 μL) contained: 10 μL 2×Hieff® PCR Master Mix (Yeasen, China), 6.4 μL ddH_2O, 2 μL DNA template (diluted to 30 ng/μL using ddH_2O), 0.4 μL each of forward and reverse primers (10 μM) for the female-specific marker and 18S rDNA. PCR reactions were carried out with the following cycling conditions: 95°C for 10 min; 35 cycles of 95°C for 15 s, 56°C for 20 s, 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were examined using 1% agarose gels, and female-specific amplicons were sequenced with the Sanger approach (BGI, China).

To assess the applicability of the sex-specific markers in other populations, gDNA was extracted from tube foot tissues of 10 female and 10 male COTS individuals (a distinct population) and subjected to PCR amplification for secondary screening following the same procedure.

Results

Summary of whole-genome re-sequencing and SNP calling

Whole-genome resequencing of 30 female and 30 male COTS individuals generated approximately 1,140.67 Gb of raw data. After quality filtering, approximately 1,116.14 Gb of clean data was retained (Table S1). The average sequencing depth reached 42.58×, with Q20 and Q30 of 98.44% and 94.34%, respectively (Table S1). These results indicated that the whole-genome re-sequencing data was of high quality and suitable for downstream analysis.

The obtained clean reads were subsequently mapped to COTS chromosome-level genome, with an average mapping rate of 97.57% (ranging from 93.67% to 99.19%) (Table S2). The GATK pipeline initially identified 23,582,039 SNP sites across the genome. Following stringent quality control, 4,806,243 high-confidence SNPs were retained for further genome-wide analyses.

Localization of a candidate sex determination region

A GWAS was performed to investigate the genetic basis of sex (a binary trait). The Manhattan plot indicated that the sex-associated SNPs were mainly located on chromosome 10 (Fig. 1a). And this 3 Mb physical region did not exhibit a higher overall SNP density compared to adjacent regions (Fig. 1b). A marked upward deviation from the null distribution was observed in the QQ plot, indicating the presence of true association signals as opposed to random genetic drift (Fig. 1c).

GWAS revealed that the sex-associated SNPs were mainly located on chromosome 10, a pattern consistently observed under both "*P*-value = 1e-5" (68.8%) and "*P*-value = 1e-8" (71.0%) thresholds (Fig. 2; Table S3). To further explore the chromosomal distribution characteristics of sex genetic differences, we calculated F_{ST} values for all identified SNPs, with loci exhibiting "$F_{ST} > 0.25$" considered to represent significant genetic differentiation. Notably, the majority of SNPs with an F_{ST} value exceeding 0.25 were enriched on chromosome 10, and a consistent distribution characteristic was also observed when a more stringent threshold ($F_{ST} > 0.5$) was applied (Fig. 2; Table S3). To gather more evidence for the sex-related divergence on chromosome 10, we identified the sex-specific SNPs (fixed for homozygosity in one sex and heterozygosity in the other). Interestingly, a total of 4,159 sex-specific SNPs were identified, all of which exhibited heterozygous genotypes in female COTS individuals and homozygous genotypes in male COTS individuals, suggesting COTS might own a ZZ/ZW sex determination system. Moreover, these SNPs were also predominantly located on chromosome 10, accounting for over 80% of these loci per unit chromosome length (Fig. 2; Table S3).

In addition, principal component analysis (PCA) was performed on chromosome-specific and genome-wide SNP sets. PCA based on SNPs located on chromosome 10 clearly distinguished 60 individuals into females and males (Fig. 3a). In contrast, PCA using SNPs on other chromosomes or genome-wide SNPs showed extensive overlap between males and females, with no clear separation (Fig. 3b, Fig. S2). These results collectively indicated that the primary genetic divergence between sexes was localized to chromosome 10.

Subsequently, we focused on chromosome 10 to identify candidate sex determination regions for COTS. The GWAS of sex identified a total of 5,571 sex-associated SNPs (*P*-value < 1e-8) on the whole genome,

of which 3,859 loci (69.27%) were located in a 11–14 Mb region on chromosome 10 (Fig. 1a, Fig. 4a). SNPs with F_{ST} -value > 0.25 were also predominantly located in this genomic region (Fig. 4b). Moreover, a substantial majority of the identified sex-specific SNPs (3,284 out of 4,159) were concentrated in this region (Fig. 4c), supporting its central role in sex determination. Thus, the 11–14 Mb region on chromosome 10 was identified as a candidate sex determination region in COTS.

To investigate whether a syntenic sex determination region exists in Asteroidea, we performed synteny comparison between the 21 chromosomes of COTS and the 22 chromosomes of *A. amurensis*. The results revealed that the two chromosome 10 regions did not have a direct one-to-one orthology relationship (Fig. S3a). Furthermore, genes within their respective putative sex determination regions (11–14 Mb on COTS LG10 and 7–12 Mb on *A. amurensis* Chr10) showed poor collinearity (Fig. S3b).

Identification of candidate sex determination genes

To identify candidate genes for sex determination in COTS, we conducted functional annotation of 5,571 sex-associated SNPs (*P*-value < 1e-8). In the list of genes potentially affected by these sex-associated SNPs, no universal master sex determination gene was identified (Table S4). However, within the candidate sex determination region, 731 sex-associated SNPs were annotated in the PKNOX2 gene, including four nonsynonymous mutations (Table S4, S5). Although such gene is not a universal sex determination gene, it might influence sexual dimorphism by regulating downstream gene networks. This potential role is supported by its significantly higher expression in the testes compared to the ovaries (Fig. S4). Furthermore, several hypothetical proteins without functional annotation were linked to a substantial number of sex-associated SNPs (Table S4, S5). Despite their unknown function, these genes might play potential roles in sex determination of COTS and warrant further investigation.

Development of female-specific markers

Following the established pipeline (Fig. S1), we performed the identification and validation of the sex-specific molecular markers in COTS. The downloaded female and male reference genomes of COTS demonstrated a high level of assembly continuity, with genome sizes of 402.64 Mb and 403.84 Mb and scaffold N50 lengths of 1,749,591 bp and 978,890 bp, respectively (Table S6). Re-sequencing reads from 5 female and 5 male COTS individuals were respectively mapped to the female and male reference genomes to identify sex-specific sequences (Table S7). After artificial screening based on the alignment of pooled data from 30 females/males, we identified 15 female-specific sequences, with a total length of 6,119 bp, whereas no reliable male-specific sequence was detected (Table 1).

Table 1
Summary of sex-specific sequences after screening.

Sequence type	Sequence number	Total length (bp)	Max length (bp)	Min length (bp)	Average length (bp)
Female-specific	15	6,119	595	317	408
Male-specific	0	0	0	0	0

Next, the two longest female-specific sequences were selected for development of female-specific molecular markers (Table 2). The visualization of read coverage across samples supported their identity as female-specific sequences (Fig. 5a). To prevent false male identification due to amplification failure, we incorporated primers targeting the 18S rDNA sequence into a multiplex PCR system for reliable COTS sex identification (Table 2).

Table 2
Two pairs of primers for verification of COTS female-specific sequences (Pri1 and Pri2) and one pair of primers for positive control (18S rDNA, Pri3).

Target sequence and location	Primer ID	Primer sequence (5'-3')	Product length	Tm
JAUNZQ010000510.1: 1087366–1087898	Pri1_F	TGTCCAAGTCATTCTGCCCA	248bp	56°C
	Pri1_R	GGATGCCAGAACGCTCAAAT		
JAUNZQ010000893.1: 19682–20276	Pri2_F	CAACCTGTCTGTGTGCACTC	396bp	56°C
	Pri2_R	GCATCACATGAGCCTCTTGG		
JAUNZQ010001007.1: 648136–649950	Pri3_F	CTTTACCAAGCGAAACTGCG	581bp	56°C
	Pri3_R	CCCAAGATCCAACTACGAGC		

To confirm whether these two molecular markers (Pri1 and Pri2) could distinguish the genetic sex of COTS, we first performed PCR using genomic DNA extracted from 10 female and 10 male re-sequencing individuals (gonad tissues) as templates. The target fragment was amplified only in female samples, with no amplification observed in males (Fig. 5b), thus demonstrating that they could reliably distinguish the genetic sex of COTS. Besides, Sanger sequencing of the amplification products from both marker regions provided further evidence for the actual presence of these female-specific fragments. To further validate the practicality of these two female-specific markers, we applied them to an additional set of 10 female and 10 male individuals using tube foot tissues (sampled from different natural waters one year later during COTS sexual maturity). The PCR results confirmed that the genetic sex identified with these markers was fully consistent with the phenotypic sex (Fig. 5c).

Discussion

Crown-of-thorns starfish are a major contributor to coral loss during population outbreaks, yet a lack of fundamental insights into the molecular processes that regulate their physiological processes impacts the development of biocontrol approaches. Targeted and sustained manual culling currently represents the most direct and effective management approach for controlling COTS outbreaks (Boström-Einarsson and Rivera-Posada, 2016; Pratchett et al., 2019; Westcott et al., 2020; Matthews et al., 2024; Skinner et al., 2025). However, the labour-intensive nature and high costs of this approach preclude it from serving as a viable long-term or large-scale solution. Semiochemical technologies have gained attention as a promising alternative, offering the potential for targeted and efficient biocontrol of COTS outbreaks (Hall et al., 2017; Motti et al., 2017; Pratchett et al., 2021b; Harris et al., 2025). A particularly promising strategy within this domain involves disrupting mass spawning events, which depends on the identification of effective sex attractants (Motti et al., 2017; Harris et al., 2025). In the present study, we identified a candidate sex determination region and developed two pairs of COTS sex-specific molecular markers based on whole-genome re-sequencing data from both sexes. These markers enable unambiguous sex identification of COTS individuals, which is a prerequisite for validating the sex-specificity of candidate attractants. This work, therefore, provides a critical foundation for applying sex attractants to control pre-spawning aggregation events of COTS.

Sex determination mechanisms in echinoderms are extremely complex and diverse. Among sea urchins, both ZZ/ZW and XX/XY sex determination systems have been reported in different species (Lipani et al., 1996; Han et al., 2021; Wang et al., 2022b; Cui et al., 2021), and similar variability appears to exist in sea cucumbers (Wei et al., 2021; Wang et al., 2022a; Wu et al., 2022; Jiang et al., 2024). The only previous study on the sex determination mechanism in a starfish (*A. amurensis*) identified female-specific sequences and sex-specific SNP genotypes, suggesting a ZZ/ZW system (Wang et al., 2025). In the present study, female-specific sequences were also identified, and all 4,159 sex-specific loci exhibited female heterozygosity, likewise supporting a ZZ/ZW sex determination system in COTS. Nevertheless, the karyotype of COTS and the potential existence of heteromorphic sex chromosomes require further investigation. Notably, GWAS results in both sea cucumbers and starfish revealed a pattern characterized by the clustering of sex-associated SNPs in certain genomic regions, alongside a broader distribution across other chromosomes (Wang et al., 2022a; Jiang et al., 2024; Wang et al., 2025). This pattern might reflect a complex multi-gene sex determination mechanism, potentially a characteristic feature in echinoderms (Jiang et al., 2024). In addition, previous studies have documented the occurrence of hermaphrodites in COTS, suggesting that sex phenotype may exhibit a degree of plasticity in this species, or that some individuals may be mosaic (Guerra et al., 2019; Pieplow et al., 2023). However, in this study, we observed no gonads containing both sperm and eggs in sampled individuals.

Multiple lines of evidence support the 11–14 Mb region on chromosome 10 as a candidate sex determination region in COTS, including the distribution of sex-associated SNPs and sex-specific SNPs, selection signatures of F_{ST} values, and PCA results based on SNPs from different sets. This finding echoes our previous work on *A. amurensis*, where a 7–12 Mb region on chromosome 10 was identified as the candidate sex determination region (Wang et al., 2025). However, no synteny was observed between the chromosomes containing the candidate sex-determination regions in the two starfish

species. This indicated that the sex determination regions in these two sea stars are not derived from a shared ancestral chromosomal region, but may have evolved independently within the class Asteroidea.

No universal master sex determination gene was identified based on the functional annotation of sex-associated SNPs, suggesting a complex multi-gene sex determination mechanism in COTS. Nevertheless, genes within the candidate sex determination region were noteworthy. For example, numerous sex-associated SNPs were identified within the PKNOX2 gene, a TALE (three amino acid loop extension) class homeobox transcription factor (Imoto et al., 2001). Given the well-established role of TALE-family transcription factors in embryonic patterning and cell fate determination (Banerjee-Basu and Baxevanis, 2001; Moens and Selleri, 2006; Bürglin and Affolter, 2016), we hypothesized that PKNOX2 might function as a key regulator in establishing sexual dimorphism in COTS. In addition, we also identified several hypothetical proteins affected by abundant sex-associated SNPs. Despite their unknown function, these genes might play potential roles in COTS sex determination and warrant further investigation.

Accurate sex identification in echinoderms remains challenging due to the general lack of conspicuous external sexual dimorphism. Current reliable methods, such as induced spawning or gonadal extraction followed by microscopic examination, are inherently constrained: they are applicable only to reproductively mature individuals during the spawning season, and are invasive in nature, altering the physiological condition of the organism or even leading to mortality (Vacquier, 2011; Paredes and Costas, 2020). Although a non-lethal alternative based on secondary sexual characteristics has been described in a sea urchin, this approach is not widely applicable across echinoderms and its accuracy is limited by sexual maturity (Brundu et al., 2023). As a result, molecular marker-based approaches for sex identification have gained popularity and been applied in various echinoderm species, particularly for those with high economic value in aquaculture (Cui et al., 2021; Wei et al., 2021; Wu et al., 2022; E et al., 2023; Jiang et al., 2024; Liu et al., 2024; Wang et al., 2025). In this study, we developed two female-specific molecular markers for COTS using NGS technology. Based on these markers, the genetic sex of COTS can be rapidly determined through a simple PCR amplification followed by agarose gel electrophoresis, requiring only a minimal tissue sample (e.g., from tube foot tissue). We believe that this accurate and non-invasive sex identification method holds promise as a crucial tool for future application. For example, it can be used to systematically monitor the sex ratio of COTS populations in key marine areas during non-reproductive periods. Besides, it would be useful for investigations into the molecular components that regulate gonad maturation. Given the pronounced spawning aggregation behavior of COTS and the differential expression profiles of chemosensory-related receptors between males and females (Babcock and Mundy, 1992; Caballes and Pratchett, 2017; Roberts et al., 2018; Caballes et al., 2021; Jönsson et al., 2022; Morin et al., 2024), the use of sex pheromones to induce aggregation and facilitate targeted removal represents a promising biocontrol strategy for COTS (Motti et al., 2017; Harris et al., 2025). The future development and implementation of this strategy will benefit significantly from the non-invasive sex identification method established in this study.

In conclusion, through high-coverage whole-genome resequencing of 30 female and 30 male COTS individuals, we identified a 11–14 Mb physical region on chromosome 10 as a candidate sex determination region, and successfully developed two pairs of PCR primers that facilitate the identification of female-specific markers. This work not only provides novel insights into the diversity of sex determination systems in echinoderms, but also establishes a genetic foundation for future research aimed at understanding the mechanisms of COTS population outbreaks and developing targeted management strategies.

Declarations

Competing interests

All authors declare no competing interests.

Ethics approval

This work focused on an unregulated invertebrate species. All animal experiments were conducted in accordance with the ARRIVE guidelines (https://arriveguidelines.org). The animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Ocean University of China (Permit Number: 20141201).

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Author Contribution

M.Y.C and G.N. conceived this study. Y.L.W., X.D., W.W. and X.X.L. prepared the samples and conducted the experiment. Y.L.W. performed the data analysis and visualized the results. Y.L.W. wrote the manuscript. M.Y.C., S.C. and G.N. revised the paper. All authors have reviewed and approved the final manuscript.

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Data Availability

The whole-genome re-sequencing data have been deposited in the Genome Sequence Archive (GSA, accession number: CRA042951), which are publicly accessible through BioProject PRJCA064069 (https://ngdc.cncb.ac.cn/).

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Figures

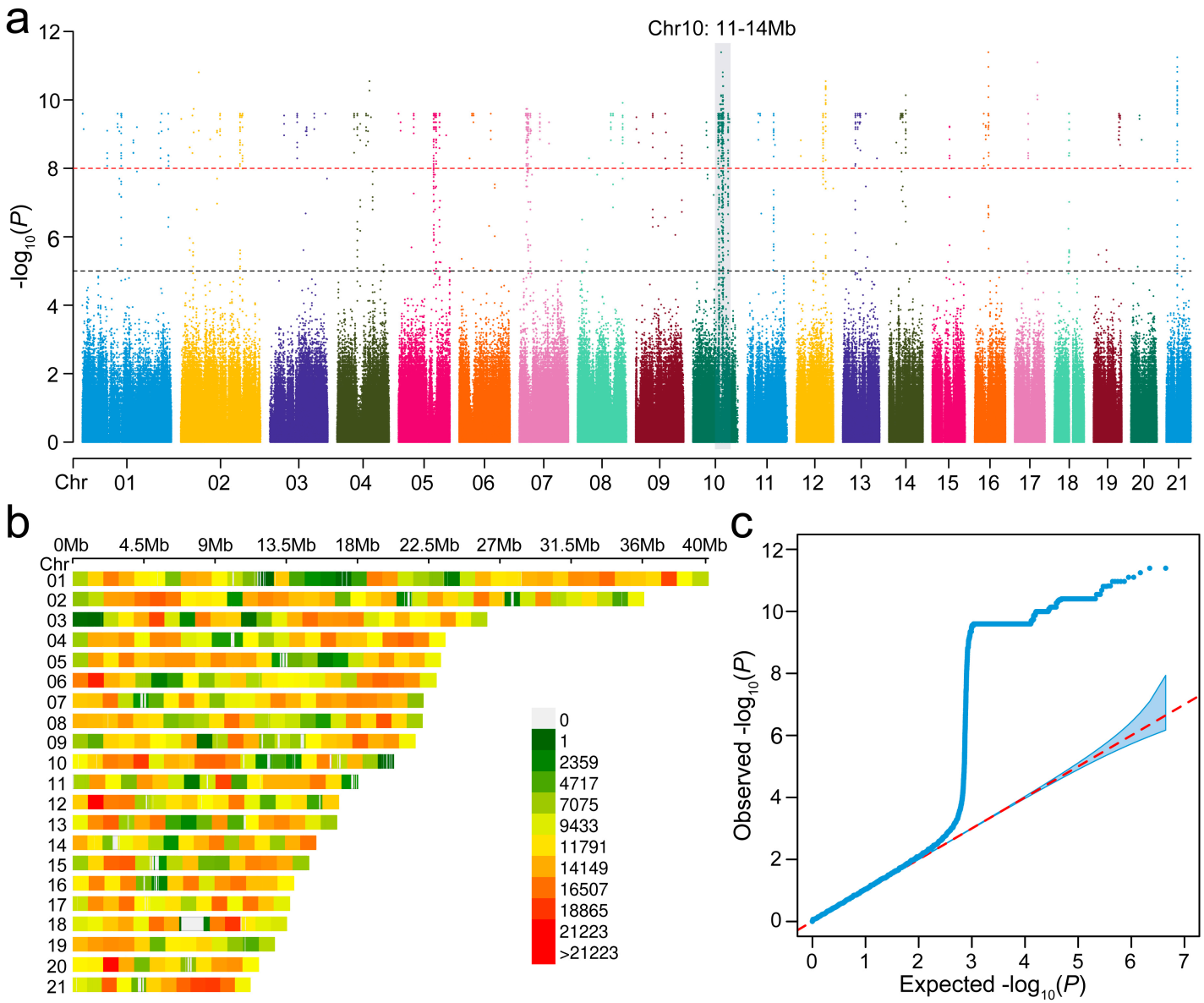


Figure 1

Whole-genome association analysis of COTS sex trait. **a** Manhattan plot. The X-axis represents the physical location of all SNPs on 21 chromosomes, and the Y-axis shows -$\log_{10}$ (*P*-value) of each SNP for

the association with sex. The black and red threshold lines represent "*P*-value = 1e-4" and "*P*-value = 1e-8", respectively. **b** SNP density plot showing the number of SNPs per Mb. **c** Quantile-quantile (Q-Q) plot. The X-axis and Y-axis represent the expected observed and the actual observed -$\log_{10}$ (*P*-value) of SNPs from association analysis. The blue region refers to the 95% confidence interval for the *P*-value distribution under the null hypothesis (no association).

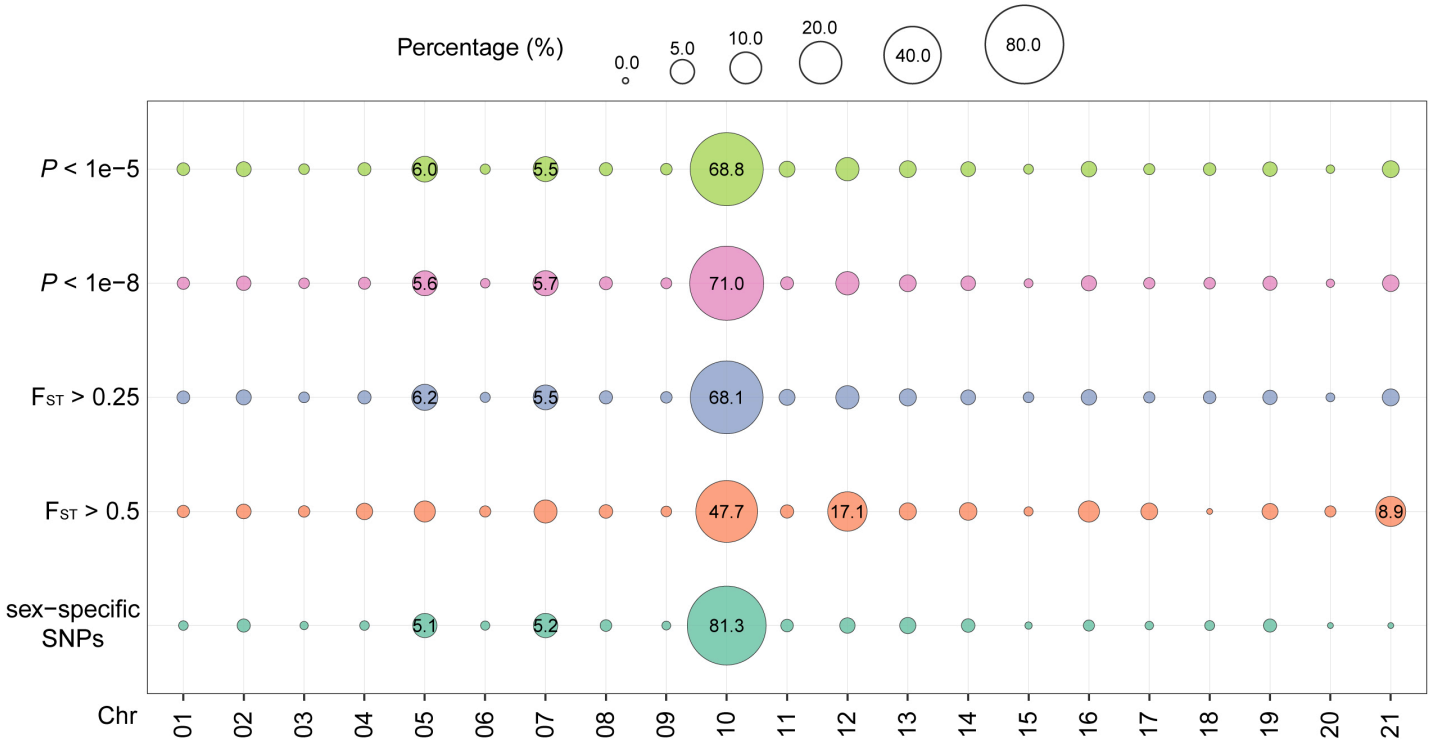


Figure 2

Chromosome distribution of the selected SNPs. The circle size indicates the percentage of SNPs per unit chromosome length for each chromosome. From top to bottom, the panels show: SNPs with *P*-value < 1e−5, SNPs with *P*-value < 1e−8, SNPs with F_{ST}-value > 0.25, SNPs with F_{ST}-value > 0.5, and sex-specific SNPs identified from the custom Perl script.

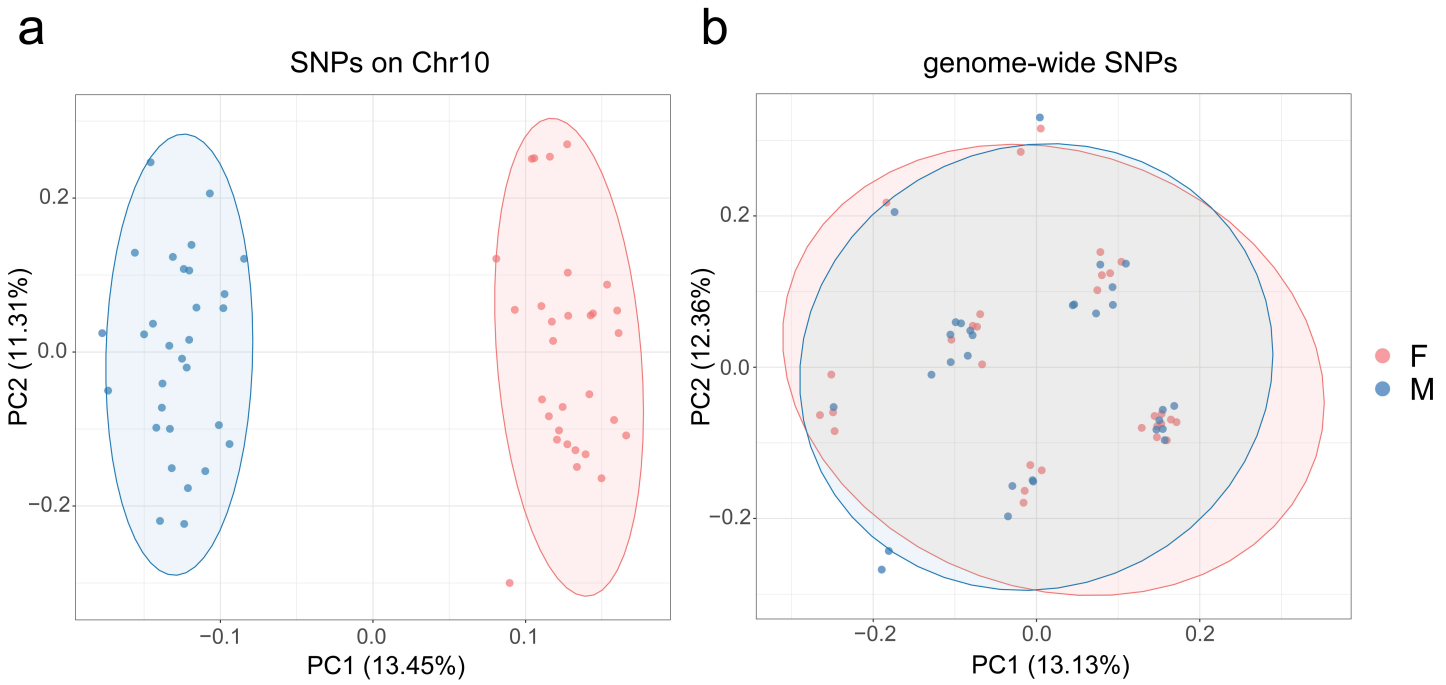


Figure 3

PCA plot based on genome-wide SNPs (**a**) and SNPs on chromosome 10 (**b**). Ellipses represent the 95% confidence region for each sex.

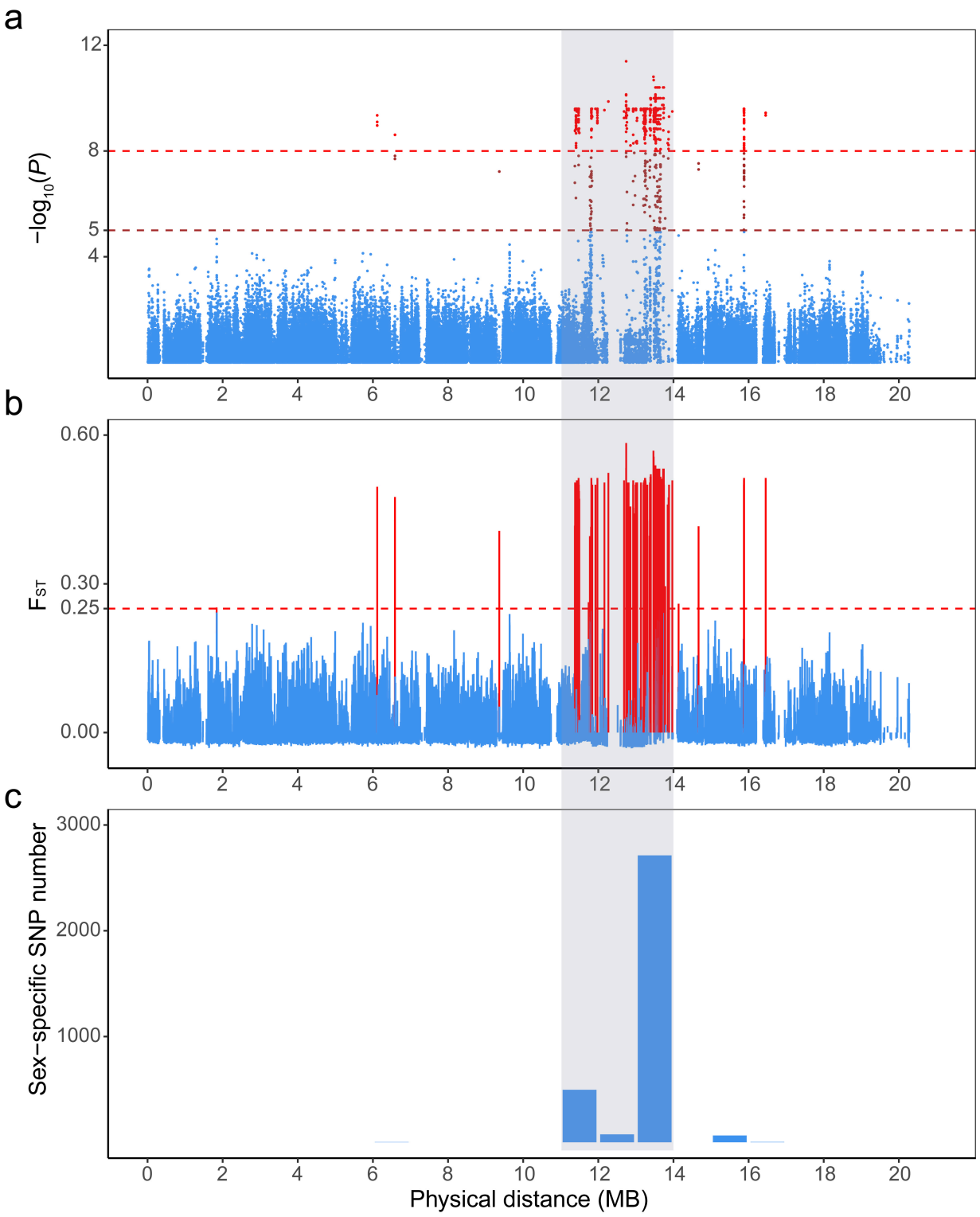


Figure 4

Identification of a candidate sex-determining region on chromosome 10 in COTS. **a** Manhattan plot for SNPs on chromosome 10. The upper and lower threshold lines represent "*P*-value = 1e-8" and "*P*-value = 1e-5", respectively. **b** Distribution of F_{ST} values for SNPs on chromosome 10. SNPs with F_{ST}-value > 0.25 are marked in red. **c** Density (per Mb) of sex-specific SNPs across chromosome 10.

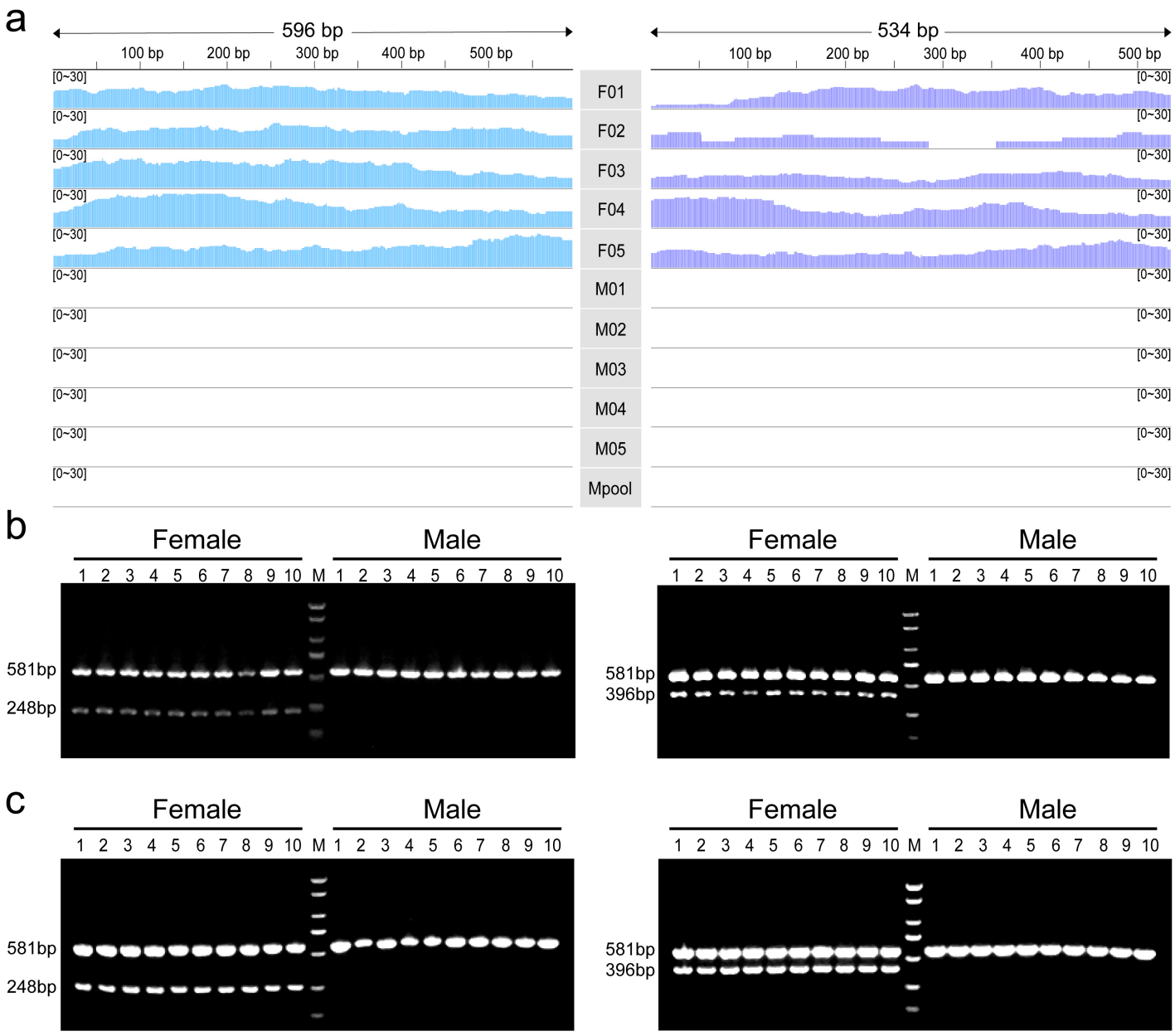


Figure 5

Screening and validation of female-specific markers. a Comparative alignment of the re-sequencing reads from both sexes on selected female-specific sequences (left: location of Pri1; right: location of Pri2). Mpool represents the pooled data of 30 male COTS individuals (M01-M30). b Primary validation of female-specific markers using gonad tissue from re-sequenced COTS individuals (F01-F10 and M01-M10). M represents DL 2000 DNA marker. The amplification product lengths for Pri1 (left), Pri2 (right) and Pri3 (positive control) are 248 bp, 396 bp, and 581 bp, respectively. c Secondary validation of female-specific markers using tube foot tissue of 10 females and 10 males from an additional population.

Supplementary Files

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