

Development and application of a sex-linked marker for *Herpetospermum pedunculosum* based on whole-genome resequencing

An-Ning Li^{1,2}, Zhi-Li Zhou², Xi-Long Wang³, Xue-Mei Wen³, Yan-Li Tu³ and Li-Hua Meng^{1*}

¹ School of Life Sciences, Key Laboratory of Yunnan for Biomass Energy and Biotechnology of Environment, Yunnan Normal University, Kunming 650500, Yunnan, China

² Yunnan Key Laboratory of Crop Wild Relatives Omics, the Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China

³ Department of Plant Resources, Institute of Plateau Biology of Xizang Autonomous Region, Lasa 850000, Xizang, China

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Sex-specific DNA markers are effective tools for sex identification and sex-controlled breeding of dioecious organisms. The seeds of the dioecious *Herpetospermum pedunculosum* are utilized in traditional Chinese medicine, and the development of sex-linked markers for seedlings is crucial for enhancing the number of female plants. In this study, we screened sex-specific markers based on whole-genome resequencing of 20 male and 24 female *H. pedunculosum* individuals, and validated a male-specific DNA fragment of 505 bp among 80 individuals from four populations using simple PCR. The findings provide a reliable male-specific marker for the sex identification of *H. pedunculosum* seedlings.

Key words: *Herpetospermum pedunculosum*, genome resequencing, indel, sex identification

Sex is a fundamental trait in both animals and plants. Most flowering plants (~90%) are hermaphroditic, with male and female functions in one flower. Dioecy, an extreme form of sexual segregation, accounts for about 6% of flowering plants (Heikrujam et al., 2015). For economically important dioecious plants (e.g., kiwifruit and pistachio), increasing the proportion of female plants potentially enhances fruit production. However, it is generally difficult to determine the sex of dioecious plant species prior to flowering, underscoring the importance of sex identification for seedlings of dioecious plants.

Sex-specific markers are valuable tools for identifying the sex of dioecious organisms (Zheng et al., 2024), and recent advancements in high-throughput sequencing technology facilitate the large-scale discovery of genome-wide molecular markers (Xu et al., 2024). Indels are widely distributed across the genome in high density and abundance (Yang et al., 2016), making them suitable as molecular markers (Gao et al., 2012). For example, indel markers have been utilized to assess genetic diversity in *Chenopodium quinoa* (Zhang et al., 2017), as well as for variety identification in *Glycine max* (Chen et al., 2021) and *Momordica charantia* (Cui et al., 2021). Additionally, indel markers are particularly effective for sex identification in fish (Du et al., 2023; Huang et al., 2024b; Zheng et al., 2024).

Herpetospermum pedunculosum (Cucurbitaceae) is a traditional Chinese medicinal plant, with distribution in southwest China, Nepal and northeast India, and its seeds are used to treat liver disease, cholecystitis and dyspepsia (Wei et al., 2020). In dioecious Cucurbitaceae species, both heteromorphic and homomorphic sex chromosomes have

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* Corresponding author. menglihua@mails.ucas.ac.cn

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been reported, and an XY sex determination system has been documented (Ming et al., 2011) in, e.g., *Trichosanthes pilosa* (Zhao et al., 2024) and *Coccinia grandis* (Janousek et al., 2022). Our unpublished genome data also strongly suggested an XY sex determination system in *H. pedunculosum*. *Herpetospermum pedunculosum* has been widely cultivated in Xizang, but wild populations of this species are strongly male-biased (ca. 70%) (Lan et al., 2011; Chen et al., 2020; Wu et al., 2024). Therefore, increasing the proportion of female plants could enhance fruit production, highlighting the importance of identifying the sex of *H. pedunculosum* seedlings.

We collected leaves of 20 male and 24 female *H. pedunculosum* individuals from a wild population in Shangri-La (27°54'36" N, 99°38'24" E, 3250 m), northwest Yunnan province. DNA of each sample was extracted for genome resequencing using the CTAB method. The quality and quantity of DNA were assessed by agarose gel (0.8%) electrophoresis, Nanodrop 2000 spectrophotometer and Qubit fluorescence spectrophotometer. The genomic DNA of each individual was randomly sheared into ~350-bp fragments with a Covaris crusher. Library preparation was performed with a Vazyme DNA library preparation kit (NDM607-01), involving end-repair, 3' adenylation, adapter ligation, purification and PCR amplification. Subsequently, paired-end sequencing was performed on the DNBSEQ-T7 platform (BGI, China).

fastp 0.12.4 (Chen et al., 2018) was used to control the quality of the raw data, and clean data were obtained with default parameters. The reads from 20 male and 24 female plants were then aligned with the reference genome using BWA 0.7.17-r1188 (Li and Durbin, 2009) with default parameters, and duplicate reads were removed using Picard MarkDuplicates (<http://broadinstitute.github.io/picard>). SNP and indel variation detection were performed using GATK 4.2.2.0 (McKenna et al., 2010).

A total of 1,995,047,592 clean reads from male plants and 1,992,527,572 clean reads from female plants were generated. For male and female plants, the average alignments were 98.09% and 99.22%, and the average coverage rates were 96.03% and 93.93%, respectively. The average sequencing depths were 17.85 for male plants and 15.04 for female plants. Nucleotide statistics revealed that the average Q20 values for clean data were 98.03% and 97.70%, while Q30 values were 94.18% and 92.94%, for male and female plants, respectively. The GC content was 38.50% for males and 38.19% for females.

To examine the genetic relationship of male and female plants, we filtered SNPs and obtained 5,204,545 high-confidence SNPs. RAXML software (Stamatakis, 2006) of the maximum likelihood algorithm was then employed to construct phylogenetic tree of 44 individuals, and admixture software (Liu et al., 2013) was used to analyze genetic structure based on a Bayesian mathematical model. The results suggested that some individuals

were closely related (Supplementary Fig. S1), and the 44 individuals could be divided into two groups, independent of sex (Supplementary Fig. S2). Therefore, the effectiveness of markers should be validated experimentally in more populations.

The indel types were annotated in Annovar software (Wang et al., 2010). The number of indels ranged from 352,581 to 1,163,931, with an average of 666,654. There were 435,721 indels in intergenic regions and 170 indels in splicing regions (Fig. 1A). The heterozygosity of indels was 55.56% and 66.28% in female and male plants, respectively (Fig. 1B), indicating a possible XY sex determination system in *H. pedunculosum*. The number of indels was high on chromosome 2 (102,930 in females and 98,330 in males) but was low on chromosome 8 (47,123 females and 40,778 in males) (Fig. 1C). Short indels of no more than 5 bp were dominant (75.0% in females and 75.2% in males), while long indels exceeding 50 bp were rare (1.38% in females and 1.37% in males) (Fig. 1D). All data related to Figure 1 can be found in Supplementary Tables S1–S4.

Genome-wide associations suggested that sites with $-\log_{10}(P)$ larger than 8 could be associated with sex (Wang et al., 2022), and we screened 954 indels located on chromosome 5 and unanchored scaffolds (Supplementary Fig. S3), which could identify the sex of at least 80% of all plants. For validation of sex-specific markers, leaves of 41 male and 39 female plants were collected in four wild populations (Table 1). Genomic DNA of each sample was extracted using the CTAB method. Primers with a length of 20 to 30 bp for indels were designed with Oligo software (Rychlik, 2007), targeting a product length of 200 to 1,000 bp. We performed PCR amplification using the following protocol: 25 μ l total reaction volume containing 12.5 μ l of 2 $\times$ SanTaq PCR Master Mix (Sangon Biotech, Shanghai, China), 10 μ M each primer, 9.5 μ l of deionized water and 1 μ l of genomic DNA. The PCR amplifications were as follows: pre-denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 52–59 °C for 30 s, extension at 72 °C for 30–60 s; and a final elongation step at 72 °C for 7 min. The PCR products were then analyzed using 1–2% agarose gel electrophoresis.

A total of 303 pairs of primers were validated, and only one pair from unanchored scaffolds successfully identified the sex of all plants across the four populations (Fig. 2). The 505-bp fragment was successfully amplified in 41 male plants but not in 39 female plants. The forward primer sequence was TAGAGGAGTGAGAAAGAGGCCGTG, and the reverse primer sequence was CGCTTCTACGCGCAATCGGTTCA. The PCR product was sequenced, and the homologous sequences in the female genome and the male genome were compared using DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA). There were three mismatched bases in the upstream primer, and 15 in the downstream primer, in the female genome (Fig. 3), which probably contributed to the failed

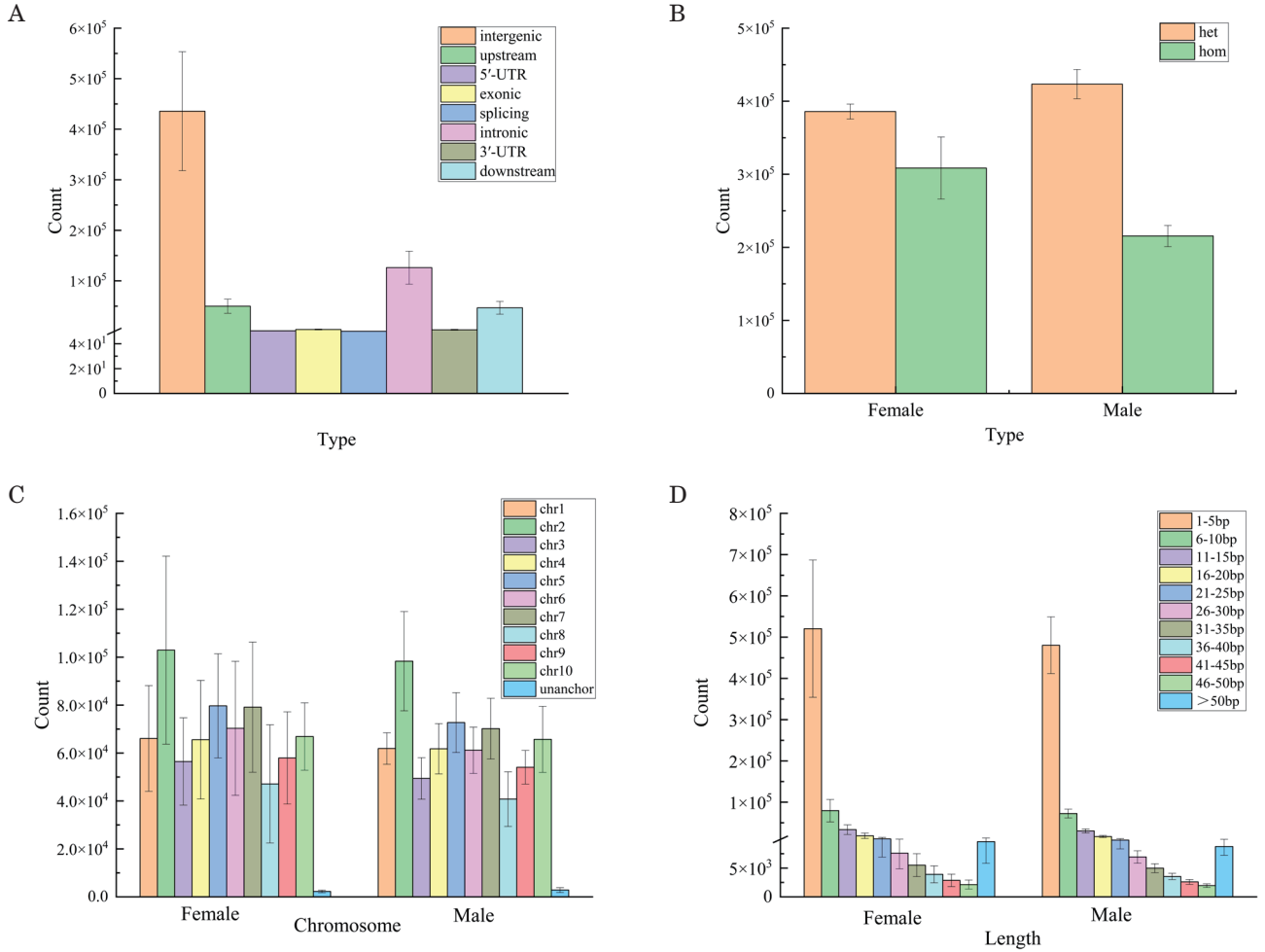


Fig. 1. General statistics of indels in resequenced male and female plants of *Herpetospermum pedunculatum*. (A) Indel type distribution. (B) Number of heterozygous and homozygous indels in female and male plants. (C) Number of indels on different chromosomes in female and male plants. (D) Number of indels with different lengths in female and male plants. Error bars indicate mean $\pm$ SD.

Table 1. Sampling sites of four wild population of dioecious *Herpetospermum pedunculatum*

Population	Location	Male	Female
Shangri-La, Yunnan	27°54'36" N, 99°38'24" E, 3,250 m	12	12
Wengshang, Yunnan	28°12'57" N, 99°44'22" E, 2,973 m	12	12
Geza, Yunnan	28°11'19" N, 99°46'48" E, 3,173 m	12	10
Chentang, Xizang	27°51'38" N, 87°25'13" E, 2,500 m	5	5
Total		41	39

PCR amplification in the female plants.

This validated sequence was homologous to the ethylene-responsive transcription factor 12-like gene (LOC111025114) in *Momordica charantia* (Supplementary Table S5). Ethylene is a key feminizing hormone in cucurbits (Switzenberg et al., 2014; Martínez and Jamilena, 2021), and the ethylene insensitive 2 gene (*EIN2*) in *Cucumis melo* inhibits the expression of the carpel inhibitor *CmWIP1*, promoting the development of female flowers (Huang et al., 2024a). The validated male-specific sequence was annotated to gene *M-21960* (with a

full length of 84,176 bp) in the male genome, and to gene *F-13860* (with a full length of 620 bp) in the female genome of *H. pedunculatum*, and a considerable number of repeats in the *M-21960* gene likely contributed to the difference in length. Between the two genes, the overall nucleotide alignment identity was 0.42% (Supplementary Fig. S4), and the overall amino acid alignment identity was 0.24% (Supplementary Fig. S5). Therefore, *M-21960* and *F-13860* may function differently in regulating ethylene synthesis between male and female plants of *H. pedunculatum*.

In conclusion, we validated a male-specific DNA marker

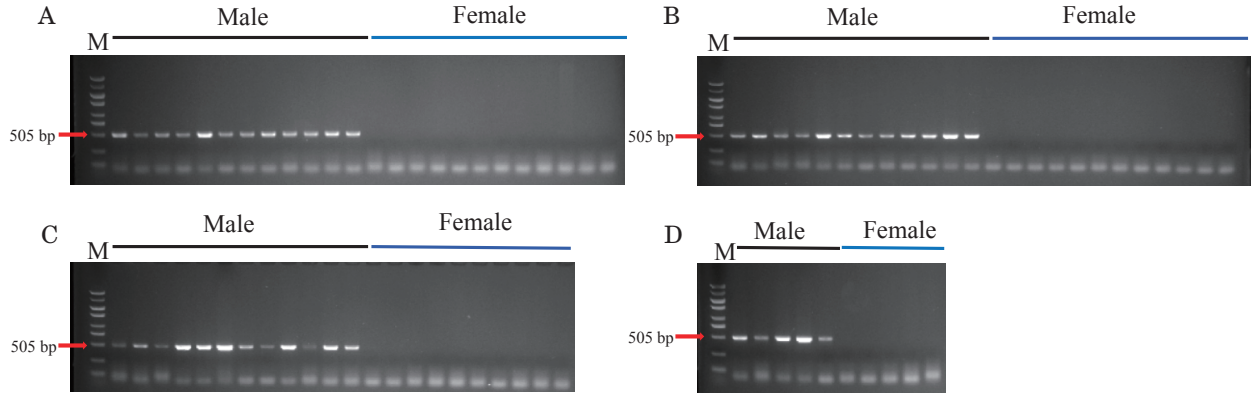


Fig. 2. Validation of the male-specific marker in all individuals from the four populations of *Herpospermum pedunculosum*. M: DL 5000 DNA marker, with bottom-up bands of 100, 250, 500, 750, 1,000, 1,500, 2,000, 3,000 and 5,000 bp. (A) PCR amplification results in Shangri-La population ($n = 24$). (B) PCR amplification results in Wengshang population ($n = 24$). (C) PCR amplification results in Geza population ($n = 22$). (D) PCR amplification results in Chentang population ($n = 10$).

Homologous sequence in female genome	ATGATGGCGTCCACTCGCGAGGGCCACT	CAGAGGCTCAGAAAGAGGCCATCGGT	CGCTACGCCCGGAGATTCCGGA	80
Homologous sequence in male genome	... ATGGCGTCAACTCGGGAGGGACACT	TAGAGGAGT GAGAAAGAGGCCGTCGGGCGTTACGCACTGAGATTCCGGA		77
Male-specific sequence		TAGAGGAGT GAGAAAGAGGCCGTCGGGCGTTACGCACTGAGATTCCGGA		51
Consensus		agagg gt agaaagaggcc tgggg cg tacgc gc gagattcgga		
Homologous sequence in female genome	CCCTTGGGAAGAAA	CCCGTGTCTGGTTAGGCACCTTCGACACTCCTGAACAAGCCGCCCTTGCTACGACGCCGCCGCC		160
Homologous sequence in male genome	TCCATGGAAGAAGACGAGGTATGGCTCGGTACTTTT	GACACACCGGAAGAGGCTGCTCATGCTTACGACGGAGCTGCCA		157
Male-specific sequence	TCCATGGAAGAAGACGAGGTATGGCTCGGTACTTTT	GACACACCGGAAGAGGCTGCTCATGCTTACGACGGAGCTGCCA		131
Consensus		cc tgggaagaa a c g gt tgg t gg acttt gacac cc gaa a gc gc c tgc tacgacgg gc gcc		
Homologous sequence in female genome	GCTCCCTCCGGGCTCC	AAGGCTAAGACCAACTTCCTCCTCCCTCC	... CTCTCCCTCGACCTCAACGTCTCC	231
Homologous sequence in male genome	GGTCTCTACGTGGTGGAAAGCAAAACT	AACITTCCTCCCGCCGATTAAAGCTGGTCTCTCCTTCGACCTTAACGTCTCC		237
Male-specific sequence	GGTCTCTACGTGGTGGAAAGCAAAACT	AACITTCCTCCCGCCGATTAAAGCTGGTCTCTCCTTCGACCTTAACGTCTCC		211
Consensus		g t c ct cg gg c aa gc aa ac aactt cc cc cc	ctctcc tegacct aacgtctcc	
Homologous sequence in female genome	ACCTGCAACTGGCATAC	CACCACCATCCACCTCCACCGCCGCGTCTGCCCACTTCTTTCACCCCGTCTCGTCTTCG		310
Homologous sequence in male genome	TCTGGATCTCACTTGTGTACCTCCCACTCCGGTCATCCCGTATCGGCGAGTTTCTTAACCGAGGTTCGCTCAAGGATG			316
Male-specific sequence	TCTGGATCTCACTTGTGTACCTCCCACTCCGGTCATCCCGTATCGGCGAGTTTCTTAACCGAGGTTCGCTCAAGGATG			290
Consensus		ctg a ct c t acc cc c cc cgg c cg cg t c c	ctc	
Homologous sequence in female genome	GCGACTTCCTCCGGCACCGCGTGCAG	GACATCAACATGTGCTTCCTTTCAATATGAATGTCAATGTGGACGCTCT		390
Homologous sequence in male genome	TGAGCTGTCCCGTCAACGAAGACCTCTCACTTCAACTAACAAAGATCTCGTTTC	TGTCTCCCTTGGCGCTAAT		393
Male-specific sequence	TGAGCTGTCCCGTCAACGAAGACCTCTCACTTCAACTAACAAAGATCTCGTTTC	TGTCTCCCTTGGCGCTAAT		367
Consensus		ga t cgg ca g g c c tcaac g t c t tgtc t g cgc t		
Homologous sequence in female genome	CCGTCTCCCGTCTCGTGGACTCGCGCTCCACCGCCACCGTACGGCGGCGTTT	TAGGCACTGCGCGGGGCAAT		470
Homologous sequence in male genome	GAA... AGCGTCAGTC	ATTCTGTGGCGTCGGAACCGTCTTCAACGACGTCGTTCTAGGCATATTTCGGCGGGGCTT		467
Male-specific sequence	GAA... AGCGTCAGTC	ATTCTGTGGCGTCGGAACCGTCTTCAACGACGTCGTTCTAGGCATATTTCGGCGGGGCTT		441
Consensus		g g gt a tc g gc c cc c c acg cg cgttt tagg c t cggcggggc t		
Homologous sequence in female genome	CCCGTTTGATCTGAACGAACCTCCACCGGTGTGGCTGCTATGA	TCAATTCAACAACTTCCCAACGG		537
Homologous sequence in male genome	GCCCTTTGATCTCAACGAACCTCCCGCGTATTGGCTG	TAAATGAACGATTGCGGTAGAACG		531
Male-specific sequence	GCCCTTTGATCTCAACGAACCTCCCGCGTATTGGCTG	TAAATGAACGATTGCGGTAGAACG		505
Consensus		cc tt t gat ct aacgaacctcc cgg tggctg t a t a c a c c a g		

Fig. 3. Sequence alignment results of the male-specific sequence and its homologous sequences in the male and female genomes of *Herpospermum pedunculosum*. Black boxes mark the positions of upstream and downstream primers, and cyan shading indicates bases with 100% identity among the three aligned sequences.

for sex identification of *H. pedunculosum*. Molecular markers based on differences in sequences are more reliable than morphological, physiological and biochemical traits (Heikrujam et al., 2015). This validated marker can accurately identify the sex of *H. pedunculosum* seedlings, which should be of great value in increasing the proportion of female plants in the cultivation of this annual medicinal herb.

DECLARATIONS

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Authors' contributions: L.-H. M. designed the study and reviewed the draft of the manuscript, A.-N. L. conducted the experiments and prepared the manuscript, Z.-L. Z. analyzed the resequencing data, and X.-L. W., X.-M. W. and Y.-L. T. collected materials from the field.

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