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Molecular Sex Identification in Dioecious *Hippophae rhamnoides* L. via RAPD and SCAR Markers

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Abstract: The dioecious property of sea buckthorn (*Hippophae rhamnoides* L.) prevents sex recognition via traditional observation at the juvenile stage, thus impeding breeding and economic cropping; RAPD and SCAR markers were used to identify the sexes. A total of 45 random decamer primers were used to screen genomic DNA pools of staminate and pistillate genotypes for genetic polymorphisms. One female sex-linked marker was identified. D15 (5′-CATCCGTGCT-3′) amplified a particular band of 885 bp, which showed polymorphism among staminate and pistillate genotype plants. The SCAR marker Hrcx-15 was obtained by sequencing the fragment. The alleles of 140 pistillate genotypes were examined but not of the 140 staminate genotypes discerned via taxonomy. Staminate and pistillate genotypes of sea buckthorn plants can be distinguished, using Hrcx-15 as a genetic marker for sex identification and for expediting cultivation for commercial applications.

Keywords: *Hippophae rhamnoides* L.; dioecious; sex determination; RAPD; SCAR

1. Introduction

Hippophae rhamnoides L. (sea buckthorn), is a multipurpose forest tree that belongs to the family Elaeagnaceae in the genus *Hippophae* L, which encompasses seven species and twelve subspecies that are widely distributed throughout the world [1,2]. Natural *H. rhamnoides* is widespread throughout the temperate regions of Asia and Europe, with distribution in the Himalayan Mountains at high altitude, and usually reproduce on sandy riverbanks or coastal dunes. Almost 90% of the world resources of *H. rhamnoides* are growing in China and the total area may exceed or approach 1.5 million hectares. *H. rhamnoides* L. subsp. Sinensis Rousi, a main subspecies and a primitive member of the genus, is endemic to China. This subspecies ranges from the northeastern part of Tibet to the southwestern corner of the Da Hinggan Mountains, through the Loess Plateau.

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Only about 6% of all 240,000 angiosperm species (14,620 of 240,000) are dioecious [3]. Gender determination of dioecy is most commonly controlled genetically by cytogenetically identified XX/XY sex chromosome system, where XY males are heterogametic sex [4,5] and females are homogametic XX [6-8]. Influenced by genetic segregation of alleles at one or several loci on autosomes [9,10], not all dioecious plants are bound to have morphologically separate sex chromosomes [11]. *Hippophae rhamnoides* L. (sea buckthorn) has a polymorphic shrubby diploid genotype with a basic composition of 2n = 24 chromosomes. Among these, 11 pairs consist of autosomes and remaining one pair are sex-chromosomes [12]. In the plant kingdom, many molecular mechanisms modulate the sex determination and many studies investigated the gender determination of dioecy. However, no definite theories exist about genetic mechanisms that control sexual dimorphism in plants.

The berries of *H. rhamnoides* taste strongly acidic and the nutritious juice is rich in vitamins (A, C, E, K, and P). In addition, clinical studies have shown that the berries boost the immune system, possess potential antitumor activity, enhance radiation protection, and improving skin problems [13-17]. However, like the majority of dioecious perennials, a unique feature of *H. rhamnoides* is that neither male nor female trees can be differentiated until the berries appear, which requires 5-7 years after seed germination [18]. Economic benefits can be increased if low-value-added male seedlings can be abandoned at the juvenile stage via apparent markers. Consequently, pistillate trees should account for the majority and an optimal proportion of 6–7% of male genotypes is considerable sufficient for providing sperm [19]. Thus, an effective and easy-to-use method to differentiate staminate from pistillate genotypes at the seedling stage is required to maintain the optimal ratio between male and female plants. Such a method has not been reported to date. Molecular bio-technology allows screening and diagnosing of gene polymorphism markers based on sex-linked DNA sequences, which can be applied long before pheno-morphological features appear. This study implemented staminate or pistillate specific genetic markers to identify the sex of *H. rhamnoides* during the vegetative growth phase.

2. Results

2.1 Sex-linked RAPD marker

Genetic polymorphism among two bulks (female and male genotypes) were screened via RAPD random decamer primers. Amplification of polymorphic bands between male and female genotypes were obtained from most RAPD primers. Among all 45 RAPD primers (decamer) screened, only one primer consistently showed a unique 885 bp fragment (D15 (5'-AATCGGGCTG-3'; Figure 1); hereafter, this was named Hrcx-15, and was particularly pronounced in the female bulk samples. This sex-linked molecular fragment was found to be reproducible. To test both the reliability and stability of the Hrcx-15, RAPD-PCR was repeated thrice, using 140 individual staminate and pistillate genomic DNAs as template. A specific band was found in all females, while this was missing in all male samples (Figure 2).

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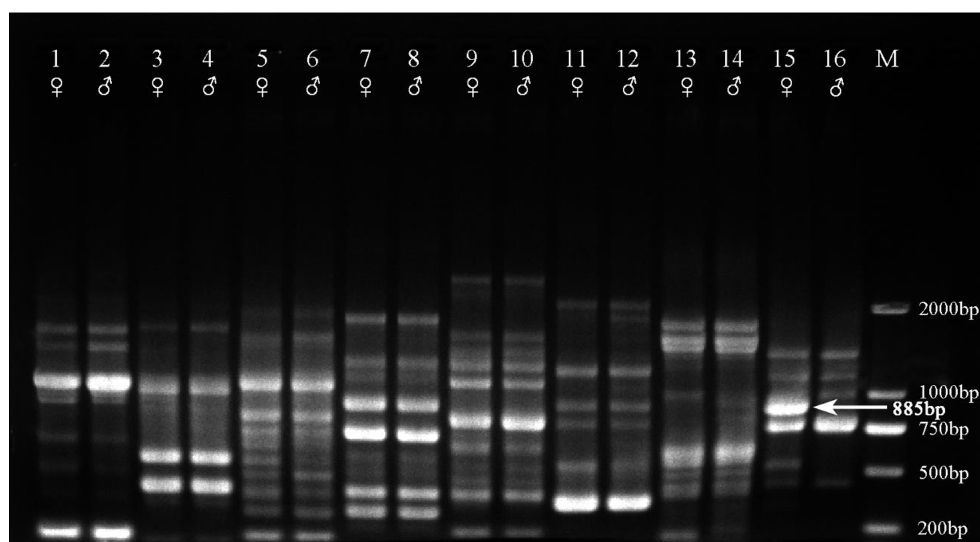

Figure 1. Part of the RAPD screening products of bulk DNA from each of the 140 male and female *H. rhamnoides* samples via decamer primers (Lanes 1, 3, 5, 7, 9, 11, 13, and 15: female; Lanes 2, 4, 6, 8, 10, 12, 14, and 16: male). The female-specific 885 bp band is indicated with an arrow. M, DNA Marker DL2,000 (Takara, China).

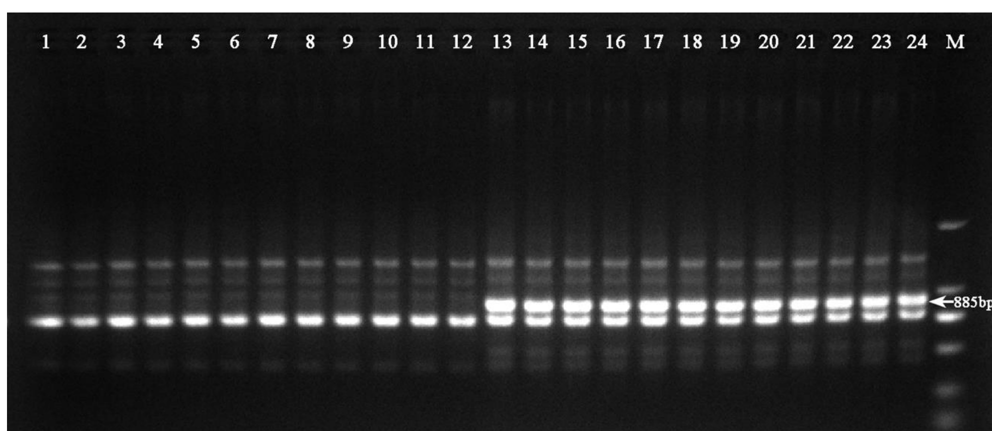

Figure 2. Amplification profile of RAPD primer D15 in *H. rhamnoides* genotypes. The arrow indicates 885 bp, lanes 1–12: male, lanes 13–24: female. M, DNA Marker DL2,000 (Takara, China).

2.2 DNA sequence of female-specific fragments

The nucleotide information (Figure 3) of specific fragments sequenced from a colony of molecular marker Hrcx-15 was submitted to the National Center for Biotechnology Information (NCBI) GenBank. The Basic Local Alignment Search Tool (BLAST) algorithm was performed to analyze DNA and protein sequence similarities with the public database stored at NCBI server. Megablast analysis of the Hrcx-15 sequence showed partial homology with the *Vitis vinifera* genome shotgun sequence AM462442.2. However, BLASTN search of the Hrcx-15 sequence demonstrated a sequence homology (E-value of 0.35, 10% query coverage) with a protein of *Frangula alnus* subsp. alnus and similarity with a protein of *Cyprinus carpio* (E-value of 6.1, 13% query coverage).

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1       CATCCGTGCT TGCATAGAAT CCGCGTGGCT TTCTGATGCG TATTTTCGCA AGTGTATGAA
61      TCGTTCAAGT AATAAAGTGT ACAAAAGTAT GGATATCGAA CCCACAAGGA ATGGCATTAC
121     TAAGTACCGA AATTGACTAA TCCTAATTTT ATTTGAAAAC CGAATTTTTG AATTTGTTTA
181     AAAAGTAAAT TAACTTAAAA CTAACGCAAA TAACAAAATT TAATGTTGTT CACAAATGAT
241     TAAAAACGCT AAGGCATTTG ATTTCGCTAA ATCAATTCAA TCCGATTTCA AGTCATGTAA
301     TATGAGAATC AAAGTTAAAA GTGATGGTTG AAAAACCCAA ATTTACCTAA TACTCTCTCT
361     CGAGTTATAT TAGAATTACT TATCTATGAA AACCGACTAC ATTTCTATGA AGATTTAATC
421     ACAAACAAGC ACATTACGAT TTATGGAATT TCTAGAATAA CCACATACAT CATGCATTAG
481     TTCTCACACT CGCATTCAAC ATACGGTATT TATCACAAGA AGCGTTCATT ACACATCTCC
541     TCTCGGTCTC AATATAATGC AACAAATCAT TTAAATCTTG ATTGATAAGA GCTAAATTAT
601     TGCATTTTTG TTATGATTTA AATGTTGATA TTTGTGTGAA AATTGTGTTT ATTTGATTAT
661     TTAATCCACA AATGTCATTT CACGGGTATT TCGTGTTTAA AGTGCAATTT CAGGTAAAAT
721     CATGATTTTG GAGAAGTTAG GGACAAAGTA CAATGATCGG GATTGAATCA AAGAAAAAGA
781     AAACAAAATT TCAAAATTTG GAATTTCGCC GCCGGCGAGA ATATTCTCGC CGCCGGTGAA
841     AACCTTACGG AGAAAATGTT GAAAATCTTC CCGAGAGCAC GGATG
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Figure 3. Nucleotide Sequence of the RARD Amplicon Hrcx-15.

2.3 SCAR marker development and examination

According to the sequence of the RAPD-PCR products that were amplified with the D15 decamer primer, a converted SCAR marker (primer combination Hrcx-15-F and Hrcx-15-R) was produced that showed a single, distinct, and bright 885 bp band in all 140 pistillate genotypes, while no products were found in the 140 staminate genotypes (Figure 4). This 885 bp amplification product was named sequence-characterized amplified regions marker Hrcx-15.

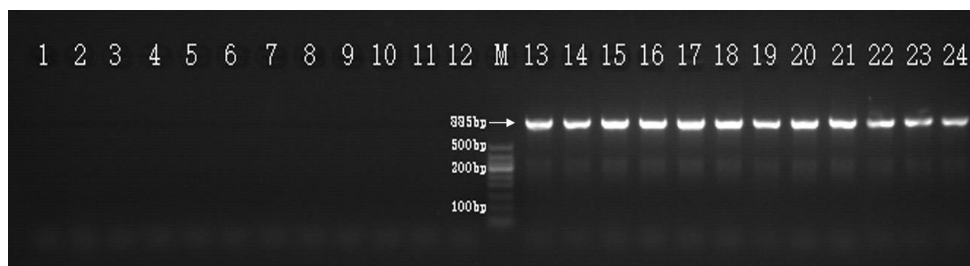

Figure 4. Amplification profile of the SCAR marker Hrcx-15 in *H. rhamnoides*, showing the 885 bp fragment, indicated with an arrow, in all female samples. Lanes 1–12: male, lanes 13–24: female. M, DNA Marker DL500 (Takara, China).

3. Discussion

Until now, reports about the genome sequence of *H. rhamnoides* are missing. RAPD analysis has the advantage to reveal a high degree of polymorphism without prior knowledge of the DNA sequence information of the species. However, to screen markers that are linked to sex determination genes on chromosomes via RAPD amplification depends on a variable success rate, because RAPD amplification is sensitive to the PCR reaction conditions with random decamer primers. In this context, we report a consistent and reproducible RAPD-PCR

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method for gender differentiation of *H. rhamnoides*.

Previously, various studies have explored sex-linked RAPD-SCAR markers in *H. rhamnoides*. According to Persson and Nybom [20], the approach of RAPD-PCR technology has been successfully applied to identify the sex of *H. rhamnoides*. Seventy-eight primers were tested and only one staminate specific marker was qualified (OPD15-600). Out of the 31 decamer primers that were used by Satender et al. [21] to differentiate pistillate *H. rhamnoides* from staminate trees, only a single primer (OPF-11 with sequence 5'-TTGGTACCCC-3') produced an 1190-bp band, which resulted in a single gender specific marker. This 1190-bp RAPD marker, which was consistently and reproducibly found in pistillate trees but was completely absent in male trees, can be used to select staminate and pistillate genotypes of *H. salicifolia*. A further attempt to recognize the gender-specific genes in *H. rhamnoides* was made by Sharma et al. [22], where the sex-linked RAPD marker OPD20-911 matched male plants. However, it is doubtful that the examination was confined to five staminate and pistillate genotypes only. Korekar et al. [23] ascertained two molecular SCAR markers linked to *H. rhamnoides* through screening 60 RAPD primers from 20 male and 20 female bulk samples. Both female-specific markers (HrX1 and HrX2) were tested via a series of pistillate (120) and staminate (100) samples.

Based on the RAPD-PCR marker D15 that was linked to female *H. rhamnoides*, a pair of 20 oligonucleotide primers was obtained to apply highly specialized PCR amplification under stringent conditions. This guaranteed to reach a more convincing conclusion for distinguishing the early gender of *H. rhamnoides*. Gender identification of seedlings at the juvenile stage prior to plantation could help to revolutionize its future cultivation, as well as to investigate the sex determining mechanism of dioecious populations. The dioecious *H. rhamnoides* has an XX/XY sex chromosome. An X/Y chromosome sex determination system has been described in a heteromorphic chromosome pair in staminate genotypes [24]. The total and relative length of the Y-chromosome surpasses X-chromosome size [25]. The development of sex-linked nucleic acid sequence makes up a vast portion of the population, as shown in this paper, which suggests that the sex of *H. rhamnoides* is likely determined genetically.

Additional markers need to be developed that are linked to gender-determination genes; this would allow the cloning of gene(s) involved in the process to generate SCAR markers. Despite the significant value of commercial plantation (allowing the differentiation of pistillate genotypes), these molecular markers would hereafter support gene localization of x-linked locus in *H. rhamnoides*.

4. Materials and Methods

4.1 Plant material

Green leaves of *H. rhamnoides* L. subsp. Sinensis Rousi were gathered from the eastern region of Qinghai Province, China (Table 1). 140 female and 140 male trees were discerned by a taxonomist during the fruiting month of August, when identification of staminate from pistillate genotypes was apparent. Green and tender leaves were plucked from both male and female trees and samples were kept at -80°C for further research.

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Table 1. Information of the collected *H. rhamnoides* L. sample in Qinghai Province

Region	Elevation (m)	Longitude	Latitude
Mayigou reservoir, Huangzhong County	2644	E101º36'26.39"	N36º29'34.76"
Dahua Village, Huangyuan County	2745	E101º1058.77"	N36º41'20.60"
Chuchuer village, Pingan County	2875	E101º51'14.50"	N36º23'33.24"
Xiangyi village, Longhua County	2933	E102º02'40.21"	N36º12'11.84"
Tangfang village, Xunhua County	2544	E102º22'34.12"	N35º49'41.65"
Tiegeleng village, Xunhua County	2840	E102º37'27.01"	N35º42'06.18"
LangTang Village, Minhe County	2636	E102º44'10.64"	N36º02'59.05"
Xiaoshuiquan village, Pingan County	2556	E101º57'57.64"	N36º29'29.05"
Pandao village, Huangzhong County	2816	E101º21'03.40"	N36º34'49.34"
Youning temple, Minhe County	3007	E102º11'36.45"	N36º45'10.16"
Bianmagou Village, Datong County	3010	E101º50'40.05"	N36º57'45.36"
Double-tree village, Huzhu County	2436	E101°55′01.91″	N36°46′47.96″
Chengguan nursery, Datong County	2566	E101°33′12.67″	N37°01′55.57″
BaoKu Township, Datong County	2683	E101°33′59.65″	N37°06′09.15″
Botanical Garden, Xining City	2314	E101°44′38.09″	N36°37'29.17"

4.2 DNA extraction

Genomic DNA from the green leaves of all trees was isolated via the Takara Plant Genomic DNA Extraction Kit after grinding plant tissue in liquid nitrogen. Both concentration and quality of genomic DNA were measured via electrophoresis or UV absorption spectrophotometer. Initially, two bulks, one from staminate genotypes and the other from pistillate genotypes, were mixed with equal mass of DNA from each of the 140 different-sexed plants.

4.3 RAPD amplification

DNA amplifications of bulk DNA alleles from all 240 plants were screened with 45 random decamer primers for RAPD analysis (Sangon Biotech, China). The thermal cycler was programmed as reported by Williams et al. [26]. The final volume of 20 μL for PCR amplification reaction contained 1U of Taq DNA polymerase (Takara, China), isolated genomic DNA 50 ng, Tris-HCl 10 mmol·L^{-1}, $MgCl_2$ 1.5 mmol·L^{-1}, 200 μmol·L^{-1} each of dNTP, and random decamer primers 25 pmol·L^{-1}. The PCR reactions were carried out in a Veriti™ 96-well Thermal Cycler and amplified using the following conditions: start with 5 min denaturation at 94°C; a total 45 cycles consisting of 1 min denaturation at 94°C followed by 37°C (1 min) for primer annealing and 72°C (2 min) for extension; and ended with an extension (7 min) at 72°C. The amplification mixtures were refrigerated at 4°C. PCR amplification products were run on 1.0% agarose gel via electrophoresis stained with 0.01% GelRed solution (Sangon Biotech, China) in 1 × TAE buffer. Gels were scanned via UV-light and photographs of were taken via Azure cSeries imaging systems (Azure Biosystems, U.S.A.). The size of DNA fragments was estimated via spotting Molecular DNA Marker DL2, 000 (Takara, China).

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4.4 Cloning of the RAPD Amplicon

The selective 885 bp polymorphism band was amplified by the decamer random primers D15, excised from 1.5% agarose gel, and was purified via Agarose Gel DNA Extraction Kit (Takara, China). The purified PCR products with an adenine tail were mixed with the sequencing vector pGEM®-T (Promega, U.S.A.) using an optimum nsert:vector ratio, and the mixtures were incubated at 4°C overnight to obtain the maximum number of transformants. The ligation reaction was added into the JM109 high-efficiency competent with 45 s heat-shock in a 42°C water bath [27]. The white positive colony was selected via blue/white color screening on indicator plate and the recombinant plasmid was obtained via overnight culture. Successful cloning of the insert was identified by amplifying the vector with the specific promoter primers SP6 and T7, which were also used for sequencing.

4.5 SCAR marker development and examination

Sequences originated from the gender specific RAPD amplicon were used to design the SCAR primers via Primer Premier 6.0 software. The primers Hrcx-15-F (5'-CATCCGTGCTTGCATAGAAT -3') and Hrcx-15-R (5'- CATCCGTGCTCTCGGGAAGA -3') for SCAR marker Hrcx-15 were designed and chemically synthesized. The PCR reaction was optimized with the following amplification conditions: totally, 30 cycles were performed, each cycle consisting of 30 s initial denaturation at 94°C, 30 s annealing at 56°C, and extension at 72°C for 1 min. The amplifications were performed with 94°C for 5 min before the start of the first cycle and ended with a 7 min final extension at 72°C after the final cycle. The amplification mixtures were investigated on 1.5% agarose gel. The designed and synthesized SCAR primers were examined via genomic DNA from 140 staminate and 140 pistillate plants.

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Author Contributions: Y.S. and N.H. conceived and designed the experiments; W.Z. performed the experiments; H.W. and G.L. revised the manuscript. G.Z. and Y.W. analyzed the data; J.M. and S.C. contributed materials; W.Z. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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