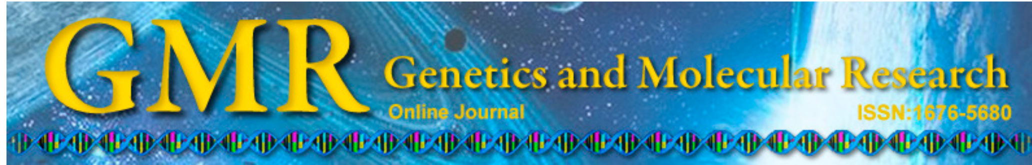

Identification of an SCAR marker related to female phenotype in *Idesia polycarpa* Maxim.

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ABSTRACT. *Idesia polycarpa* Maxim. is a dioecious species. Because of the lack of morphological and cytological methods available for identifying its sex during the long juvenile stage, the application of molecular markers in sex identification may facilitate sex determination in the seedling stage. The objective of this study was to use sequence-related amplified polymorphism to identify sex-linked markers in *I. polycarpa* and convert these markers into sequence-characterized amplified region markers, which are much easier to identify. A total of 342 primer combinations were screened and 2770 bands were examined. Only me14/em8 could amplify a specific fragment (210 base pairs) in all female but none in male plants. We analyzed this fragment using GenBank and found that the sequence similarity was 80% to the *Populus trichocarpa* clone POP006-H09 (sequence ID: gb|AC212923.1|) and that of the deduced amino acid sequence was 73% to the integrase of *Mendicago truncatula* (sequence ID: gb|ABD28291.1|) and 71% to the predicted retrotransposon integrase-like protein 1-like in *Cicer arietinum* (sequence ID: ref|XP 004515460.1|) (NCBI database through December 17, 2013). This fragment was converted into a stable and simple sequence-characterized amplified region marker approximately 200 base pairs in length. This marker can be utilized for early sexual identification in *I. polycarpa*, which will facilitate future breeding programs.

Key words: Dioecious; *Idesia polycarpa* Maxim.; SCAR; Transposition; Sex determination; SRAP

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INTRODUCTION

Idesia polycarpa Maxim. is a dioecious tree of the Flacourtiaceae family. This tree is native to some Asian countries, including Korea, Japan, and China (Kim et al., 2005). In China, *I. polycarpa* is widely cultivated in the provinces to the south of Qinling Mountain and Huaihe River. Additionally, because of its adaptability and beautiful appearance, *I. polycarpa* is an ideal plant for gardeners in northern China. The fruit of this plant has also been used to prepare edible oil (Yang et al., 2009), which contains a variety of compounds such as idesolide, which may be useful in combating obesity (Hwang et al., 2012). As an energy plant, the female trees are more valuable than male trees for practical application. The tree flower from May through June, depending on the temperature, when it is possible to easily determine the sex of the tree. However, the reproductive maturity of seedlings takes 5 or 6 years, making it difficult to distinguish the male and female plants. There have been no previous studies examining the sex chromosomes of male and female individuals. Thus, predicting the sex of an *I. polycarpa* plant before the flower buds mature is difficult. A method for determining the gender of this plant during the seedling stage would facilitate selection and breeding.

Although 10% of flowering plants produce unisexual flowers, relatively little information is available regarding the genetic basis of sex determination. Many studies focused on sex determination in plants (Adam et al., 2011), but most plant species do not have distinct sex chromosomes. Therefore, cytological methods cannot be used to identify sex in most dioecious plants. Many dioecious plants have been successfully used for studying sexual determination, such as *Asparagus officinalis* L. (Jiang and Sink, 1997), *Eucommia ulmoides* Oliv. (Wang et al., 2011), and *Buchloe dactyloides* (Zhou et al., 2011), among others.

In recent years, various molecular marker systems have been developed and used for the study of sex identification in plants. These methods include restriction fragment length polymorphism (RFLP) (Nelson et al., 2005), random amplified polymorphic DNA (RAPD) (Samantaray et al., 2010), amplified fragment length polymorphism (AFLP) (Witkowicz et al., 2003), and simple sequence repeats (SSR) (Markussen et al., 2007; Pakull et al., 2011). RFLP is time consuming and labor intensive. Although AFLP provides a high multiplexing ratio, high DNA quality is required and this method is complex. SSR is one of the most widely used marker systems because of its extensive dispersion around the genome and because it produces co-dominant markers, but it is expensive to develop. Although, RAPD is the most widely used molecular marker for sex identification, its low reproducibility limits its effectiveness (Zhou et al., 2011). These techniques generate relatively complex patterns for identification purposes and make the analysis of DNA fragments tedious and time consuming. Therefore, developing efficient methods for easy and accurate identification in routine procedures is imperative. The sequence characterized amplified region (SCAR) was first derived from RAPD fragments (Paran and Michelmore, 1993). It has been recently developed for various identification applications (Naqvi and Chattoo, 1996; Gunter et al., 2003). However, few RAPD markers can be transformed into SCARs (Vidal et al., 2000).

In contrast, the sequence related amplified polymorphism (SRAP) marker system was first described by Li and Quiros (2001). It is based on 2-primer amplification. The forward primers amplify exons to open reading frame regions, while the reverse primers target promoter and intron regions. Compared with other systems, SRAP has several advantages: it is easier than RFLP, simpler than AFLP, less expensive than SSR, and more stable than RAPD. Most importantly, it allows for the easy isolation of DNA fragments for sequencing. Thus, by

cloning the amplified bands, sequencing the ends, and then using the sequences to generate extended oligomer primers, SRAPs can be converted into stable and reliable markers. However, there is little information regarding the use of SRAP for sex identification and only in a few plant species (Zhou et al., 2011).

Thus, the goal of this study was to develop a reproducible, convenient, and reliable sex marker for *I. polycarpa* Maxim. Here, we describe a female-specific SRAP marker and its conversion into a SCAR marker. According to the sequence analysis of the SRAP marker, we predicted the development of sex in *I. polycarpa* plants.

MATERIAL AND METHODS

Plant materials and DNA extraction

All trees studied were distributed in 5 different regions in Shannxi Province (Table 1). We located 11 male and 16 female trees in Hanzhong. In Ankang, there were 10 male and 9 female trees. In the nursery of Northwest A&F University, there was 1 male and 5 female trees.

Genomic DNA was isolated according to the method described by Porebski et al. (1997) with minor modifications. Young leaf tissues (0.5 g) were ground in liquid nitrogen and 1 mL 2X cetyltriethylammnonium bromide extraction buffer. The tube was incubated for 45 min at 65°C and then centrifuged at 12,000 rpm for 10 min. To the supernatant, 1 mL chloroform/isoamyl alcohol (24:1) was added and the sample was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred and one-tenth of the aqueous layer volume of 3 M sodium acetate and an equal volume chilled ethanol were added. The DNA precipitate was washed twice with 75% (v/v) ethanol and dissolved in 200 µL ultrapure water. The DNA was quantified on a 1.0% agarose gel by visual comparison with known quantities of DNA ladder (CWBio, CoWin Bioscience Co., Ltd. Beijing, China). The DNA concentration was determined by an UV spectrophotometer. The DNA was diluted to a final concentration of 200 ng/µL. All genomic DNA was stored at -20°C until use.

DNA samples from 30 females and 22 males were mixed in equal volumes to construct 2 DNA pools.

Table 1. Geographic location and sample size of male and female trees.

Location	Sample size		Approximate geographic location		
	Male	Female	Latitude (N)	Longitude (E)	Altitude (m)
Ningqiang county, Hanzhong	2	4	32°37'-33°12'	105°21'-106°35'	620-1000
Shujiaba county, Hanzhong	9	12	32°50'-32°55'	105°25'-106°07'	1000-1800
Langao county, Ankang	9	9	32°17'-32°30'	108°54'	1020-2100
Ziyang county, Ankang	1		32°31'	108°32'	477
Nursery of Northwest A&F University, Yangling	1	5	34°15'	108°39’	563

SRAP analysis

SRAP analysis was carried out according to the method described by Li and Quiros (2001), Ferriol et al. (2003), and Li et al. (2003). A total of 342 combinations of 18 forward primers and 19 reverse primers (Life Technologies, Carlsbad, CA, USA) were used (Table 2).

Each amplification reaction was carried out in a 25-μL volume that included 1 μL 200 ng/μL genomic DNA, 0.3 μM (each) forward and reverse primer, 2.5 U *Taq* polymerase, 250 mM dNTPs, 1.5 mM $MgCl_2$, and 1X polymerase chain reaction (PCR) buffer. The reaction was completed using the following cycle parameters: denaturation at 94°C for 5 min; 5 cycles of 1 min at 94°C, 1 min at 35°C, and 1 min at 72°C; 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and an annealing temperature at 72°C for 8 min. PCR products were analyzed by electrophoresis on 6% polyacrylamide gel using a vertical-gel apparatus (LiuYi, Beijing, China) in 1X Tris/boric acid/EDTA buffer at 250 V constant current for 2.5 h. Amplified DNA fragments were detected using the silver-straining method.

Table 2. Primer sequences of polymorphic SRAP markers used in this study.

	Forward primers (5'→3')		Reverse primers (5'→3')
me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
me5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTAAC
me6	TGAGTCCAAACCGGTAA	em6	GACTGCGTACGAATTGCA
me7	TGAGTCCAAACCGGTCC	em7	GACTGCGTACGAATTCAA
me8	TGAGTCCAAACCGGTGC	em8	GACTGCGTACGAATTCTG
me9	TGAGTCCAAACCGGACA	em9	GACTGCGTACGAATTCAC
me10	TGAGTCCAAACCGGACG	em10	GACTGCGTACGAATTCAT
me11	TGAGTCCAAACCGGACT	em11	GACTGCGTACGAATTCTA
me12	TGAGTCCAAACCGGAAC	em12	GACTGCGTACGAATTGTC
me13	TGAGTCCAAACCGGATG	em13	GACTGCGTACGAATTCTC
me14	TGAGTCCAAACCGGAGT	em14	GACTGCGTACGAATTCAG
me15	TGAGTCCAAACCGGTAG	em15	GACTGCGTACGAATTGAT
me16	TGAGTCCAAACCGGTTG	em16	GACTGCGTACGAATTGAG
me17	TGAGTCCAAACCGGTGT	em17	GACTGCGTACGAATTGCC
me18	TGAGTCCAAACCGGCAT	em18	GACTGCGTACGAATTTCA
		em19	GACTGCGTACGAATTACT

SCAR marker development

The fragment of sex-specific was excised from polyacrylamide gel electrophoresis and extracted using the Long Range Gel Extraction Kit (CWBio). A total of 1 μL extraction product was used as a template in the 25-μL PCR mix (as used for SRAP amplification, above) which contained the same primer combination. PCR products were separated by electrophoresis on a 2.0% agarose gel. The selected fragment was excised from the gel and purified using the UNIQ-10 EZ Spin Column DNA Gel Extraction Kit (Sangon Biotech Co., Ltd., Shanghai, China). The DNA band was ligated into the pUC-T cloning vector using cloning kit (CWBio). Recombinant plasmids were transformed into *Escherichia coli* strain (DH5α) using the heat shock method. The cloned DNA fragment was sequenced by Life Technologies Inc. The sequence was analyzed using NCBI BLAST algorithms (http://www.ncbi.nlm.nih.gov).

SCAR primers were designed according to the sequence of the cloned SRAP fragment. The annealing temperature of these primers was measured using the software Oligo 7.0. The SCAR reaction was carried out in a 25 μL mixture using the following cycling parameters: 1 cycle for 4 min at 94°C; 30 cycles for 1 min at 94°C, 1 min at the annealing temperature, and 2 min at 72°C; followed by final extension at 72°C for 7 min. The PCR products of

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each sample were examined on a 2.0% agarose gel to determine whether the SCAR primers had been converted successfully.

RESULTS

In this study, 342 primer combinations were used to identify the sex-specific SRAP marker in the 2 DNA pools. Of these, 308 combinations generated a total of approximately 2770 fragments, ranging from 50-2000 base pairs (bp), and 76 pairs of potential primers produced 109 DNA fragments that differed between the 2 DNA pools. Only 1 pair of primers amplified a stable and specific fragment in all female samples, but none in the males when each of the 30 female and 22 male individuals was tested (Figure 1). The sex-specific fragment (210 bp) in the female pool was amplified by primers me14 and em8. This result was tested at least 3 times using the same procedure and the amplification pattern was found to be consistent.

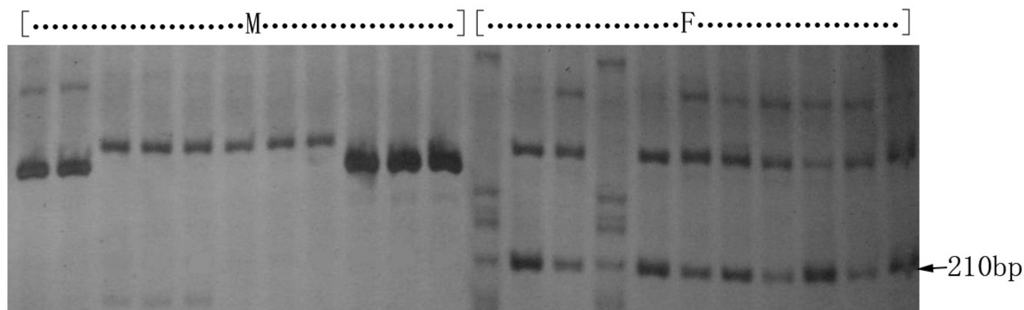

Figure 1. SRAP band patterns from DNA extracted from 11 male (**M**) and 11 female (**F**) individuals using primer combination me14/em8. The specific fragment (210 bp) is indicated by an arrow.

The sequence of the cloned female-specific fragment was determined (Figure 2). The sequence was evaluated using GenBank and the sequence similarity was 85% to the *Populus trichocarpa* clone POP021-E24 (sequence ID: gb|AC216545.1|). Additionally, pairwise alignment of the deduced amino acid sequence of this fragment revealed that the sequence similarity was 73% to the integrase of *Medicago truncatula* (sequence ID: gb|ABD28291.1|) and 71% to the predicted retrotransposon integrase-like protein 1-like in *Cicer arietinum* (sequence ID: ref|XP 004515460.1|) (NCBI database through December 17, 2013).

The primers TZS1 and TZS2 were designed based on the sequences (Figure 2). The optimized annealing temperature of the SCAR primers was 60°C. At this annealing tempera-

TGAGTCCAAA CCGGAGTAAA TGGGTACCAA ACTATGAGGG TCCTTATATA
TZS1
GTGAAGAAAG CTTTTTCAAT AGAAGCCCTA ATATTGTTTA GCATGGATGG
AGAAGATTTA GCCAGACCTG TGAATTCTGA TTCTATGAAG AAATACATGT
ATGATGAGGT GTTTCTTAGT CGATTGGTT GTGGTTAAATC CCCTGAATTG
CAATTCAGGGG ATTTAACCAC
TZS2
GTACGCAGTC

Figure 2. Sequence of the female-specific marker (210 bp). The boldfaced underlined nucleotides define the forward and reverse SCAR primers (TZS1 and TZS2).

ture, a SCAR marker was obtained (~200 bp). The agarose gel in Figure 3 shows that the marker was observed in all the female individuals, but in none of the male plants. Thus, the female-specific SRAP marker was successfully converted into a SCAR marker.

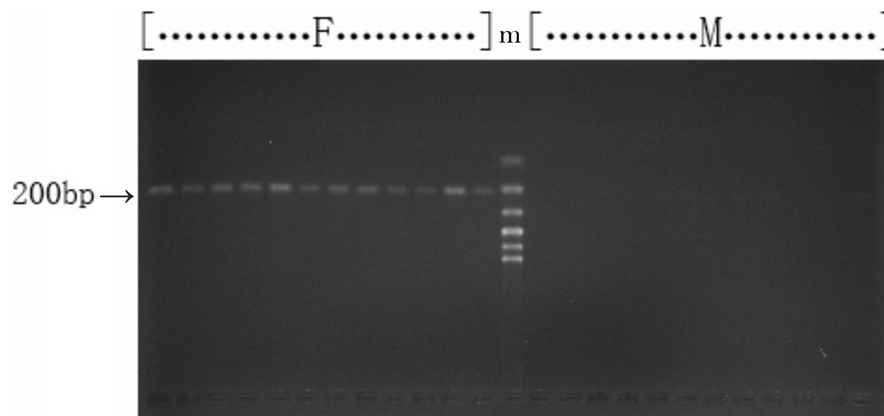

Figure 3. SCAR marker linked to sex in *Idesia polycarpa* Maxim. analyzed using the primer combination TZS1 and TZS2 of individual plants obtained from the female plant (arrow). A band approximately 200 bp was observed in the females (**F**) but not in the males (**M**). A 100-bp DNA ladder is shown in *lane m*.

DISCUSSION

Currently, there is no reliable method for identifying the sex of *I. polycarpa* seedlings in early stages. In this study, we used the SRAP marker system to identify sex-specific fragments. Zhou et al. (2011) previously found a female SRAP marker (~240 bp) in *B. dactyloides*. Because it is much easier to sequence and convert an SRAP into a SCAR marker, these markers can be used as new research tools for sex identification (Vidal et al., 2000). SCAR marker can be easily amplified by PCR and are easily detected on agarose gel electrophoresis.

The development of unisexual flowers has long been used as a model system for understanding the mechanism of plant sex determination (Bai and Xu, 2012). Several researchers have attempted to determine the molecular mechanisms of sex determination in dioecious plants. However, most plant species do not have distinct sex chromosomes. Therefore, cytological methods cannot be used to identify sex in most dioecious plants; however, molecular markers show potential for this purpose. According to Chuck (2010), the development of unisexual flowers appears to have occurred several times independently, suggesting that plants may have evolved different sex-determination mechanisms. Martin et al. (2009) showed that the insertion of a transposon (and consequent DNA methylation changes) in the promoter of a zinc finger transcription factor gene, CmWIP1 resulted in the transition from male to female flowers in gynoecious lines of melon. In this study, we found a female-specific SRAP marker, which showed homology to some database sequences in BLAST and BLASTn searches, including 73% to the integrase of *M. truncatula* and 71% to the predicted retrotransposon integrase-like protein 1-like in *C. arietinum*. However, the specific fragment we identified was short, which may have increased the similarity with other DNA or protein sequences. However, these results may also indicate that a transposable mobile element is responsible for sex development in *I. polycarpa*. In fact, according to the farmers living in Qinling Mountain,

some *I. polycarpa* male trees (already attaining reproductive maturity) produce a few seeds in autumn. However, many other different genotypes of female and male individuals in dioecious plants limit the accurate detection of DNA methylation sites and expression levels. Thus, further studies should be conducted to determine whether this phenomenon exists and it is related to transposon behavior.

In this study, we attempted to identify 30 male and 30 female trees, but only identified 30 females and only 22 males. The sex ratio of *I. polycarpa* in the 5 locations in our study was female-biased. Dioecious plant species make up approximately 4% of all plant species (Ainsworth, 2000), and often show variation in the local proportion of male-to-female plants. Sex ratios are genetically important to plant populations. Population sex ratio of *I. polycarpa* is of direct interest to humans, as the production of fruit is a primary goal. Thus, further studies should be conducted to determine the sex ratio of other *I. polycarpa* populations and the mechanisms affecting these ratios.

This is the first study to describe the transformation of a specific SRAP marker into reliable SCAR marker for use in sex identification of *I. polycarpa*. However, we did not analyze the accuracy of the SCAR marker in a larger population. Our SCAR marker may be linked with the loci of the gender we are interested in studying. Subsequent studies will be conducted to test the accuracy of the SCAR marker in a larger population.

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