VALIDATION AND DETECTION OF SEX-SPECIFIC MARKERS IN JOJOBA (*SIMMONDSIA CHINENSIS*) PLANTS IN SAUDI ARABIA

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Abstract. The main target of the present study is the validation of sex-specific markers previously described and the detection of new markers for further use in breeding programs of jojoba in Saudi Arabia. The used molecular approaches included random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), Cleavage-amplified polymorphic sequence (CAPS) and amplified fragment length polymorphism (AFLP). RAPD and ISSR analyses were not useful in determining the sex of jojoba. CAPS with the J888 marker indicated the presence of two new sex-specific SNPs and consequent presence of several sex-specific markers. AFLP analysis successfully resulted in the recovery of several male- and female-specific markers. Whole genome sequencing was also done to detect new and validate already-known sex-specific markers. Use of NUCmer module resulted in no new sex-specific markers. BLAST analysis to validate sequence-related amplified polymorphism (SRAP) marker indicated its existence. Analysis of the possible functioning of *OGI/MeGI* sex determination system in jojoba was not proven. In conclusion, a number of sex-specific markers were detected in jojoba plants growing in Saudi Arabia might help promoting the biofuel production industry in Saudi Arabia and the Arab region as a new approach to be adopted in the near future.
Keywords: *RAPD, CAPS, AFLP, WGS, BLAST, NUCmer, SRAP, OGI/MeGI*

Introduction

Biofuels can be either bioalcohol or biodiesel. The latter is made of renewable resources that are generated from plants like jojoba and jatropha (Pinzi et al., 2009). Comparing biofuels to fossil fuels indicates that the first reduces pollution and greenhouse effects. Jojoba (*Simmondsia chinensis* (Link) C. K.) is a perennial and plant native to Arizona, southern California and northern Mexico (Benzioni, 1995). The plant is dioecious, e.g., bearing male and female flowers on different plants with the axillary inflorescences of male plants harbor a number of 3-20 flowers, while axillary inflorescences of female plants harbor single flower (Ince and Karaca, 2011). The use of jojoba in biofuel production is advantageous because the plant is non-edible, unlike corn or sugarcane, and can be irrigated with low quality water and cultivated in areas with high levels of drought, salinity and heat. Jojoba possesses unique favorable properties, such as low acidity and viscosity, good oxidation stability and its oil possesses < 3% triglyceride and is highly resistant to oxidation (El-Mallah and El-Shami, 2009). Jojoba oil has several applications that make it economically feasible (Passerini and Lombardo, 2000). It is used in lubricants, pharmaceuticals and cosmetics, besides its potential use in industry, e.g., plastics, printer ink, surfactants and leather lubricants (Inca and Karaca, 2010).

The most common method for jojoba breeding is the non-sexual propagation along with the selection of plants with desirable characteristics (Vaknin et al., 2003; Tobares et al., 2004; Benzioni et al., 2005). However, there is a shortage of yield with the use of

clones or cuttings in commercial plantations, therefore, the application of supplemental pollination results in an increased seed yield (Coates et al., 2006). Further analysis indicated that selection of appropriate male and female genotypes for plantation and breeding studies is mandatory. However, further recovery of low yield results mainly from the high female to male plant ratio (Benzioni and Ventura, 1998; Coates et al., 2006). The plantation with seeds usually results in 84% male and 16% female plants whereas only 10% male plants are desirable for optimal yield (Agarwal et al., 2008). This ratio can be easily manipulated by planting male plants distantly far from the existing females. For economical plantation of jojoba, there is a need to identify the sex of the plant at the seedling stage. Early identification of sex will also lead to increased efficiency of a plantation program by allowing proper layout of field arrangement of male and female seedlings and early elimination of unwanted plants.

Unfortunately, sex can be determined morphologically, as indicated earlier, at mature stages when plants are 3-year-old or older, while sex is difficult to be determined at earlier stages. However, sex in young seedlings was determined at the molecular level so far but with less success. Therefore, it is important to find effective molecular or DNA markers that can reliably be used in the easy determination of sex at early stages of plant development. DNA markers have several advantages as they exhibit high allelic variation, being neutral to the environmental conditions (Tan et al., 2003; Karaca et al., 2004), besides being used in sex identification in several plant crop species including basket willow (*Salix viminalis* L.) (Alstrom-Rapaport et al., 1998), *Atriplex garrettii* (Ruas et al., 1998), *Viola pubescens* Aiton (Culley and Wolfe, 2000), nutmeg (*Myristica fragrans* Houtt.) (Shibu et al., 2000), *Pistacia* sp. (Kafkas et al., 2001), *Mercurialis annua* (Khadka et al., 2002), hemp (Torjek et al., 2002), *Eucommia ulmoides* Oliv (Xu et al., 2004), hop (*Humulus lupulus* L.) (Danilova and Karlov, 2006), *Encephalartos natalensis* (Prakash and van Staden, 2006), *Carica papaya* (Chaves-Bedoya and Nunenz, 2007), *Carica papaya* (Gangopadhyay et al., 2007), bermudagrasses (*Cynodon* spp.) (Karaca and Ince, 2008), subtropical carrot (Jhang et al., 2010), *Calamus simplicifolius* (Li et al., 2010) and jojoba (Mohasseb et al., 2009; Ince et al., 2010; Agarwal et al., 2011; Ince and Karaca, 2011; Heikrujam et al., 2014; Kumar et al., 2019).

The present study aims at validating a number of sex-specific markers previously used in jojoba towards further usage in breeding programs of jojoba growing in Saudi Arabia. Results of this study might help determining sex in jojoba more efficiently, thus, promoting the biofuel production industry in Saudi Arabia and the Arab region, a new approach possibly adopted in the near future.

Materials and methods

DNA extraction

The analysis involved a number of five male and five female plants growing at King Abdulaziz Farm station at Hada El-Sham near Jeddah, Kingdom of Saudi Arabia. Flash-frozen leaf materials from individual plants were crushed into a fine powder in a microcentrifuge tube using a sterilized metal rod and DNAs were isolated from leaves following the modified procedure of Gawel and Jarret (1991). Isolated DNAs were, then, treated with RNase A (10 mg/ml) and incubated at 37 °C for 30 min to remove RNA contaminants. DNA concentrations were estimated by measuring optical density at 260 nm according to the equation: DNA concentration (ug/ml) = OD260 X 50x dilution factor.

Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analyses

For RAPD analysis, a number of 20 random 10*mer* primers (Operon Technology, USA) from groups A, B and C was used in determining sex in male and female jojoba plants (*Appendix 1*). PCR was carried out in 25 µl reaction volume containing 1x PCR buffer, 4 mM MgCl$_2$, 0.2 mM dNTPs, 20 pmole primer, 2 units Taq DNA polymerase and 25 ng template DNA. PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany), programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 37 °C for 2 min, and an extension step at 72 °C for 2 min, followed by a final extension cycle for 7 min at 72 °C.

For ISSR, 14 primers were used in detecting polymorphic bands between male and female plants (*Appendix 1*). PCR analysis was performed in 25 µl reaction and amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 40 °C for 80 s, and an extension step at 72 °C for 2 min, followed by a final extension cycle for 7 min at 72 °C. Amplicons were run on agarose gel (Wide Mini-Sub Cell GT Systems, Bio-Rad) at 120 V/50 mA (PowerPac Universal Power Supply, Bio-Rad) for 45 min, then gel stained with ethidium bromide (0.3 ug/ml) and visually examined with an UV transilluminator and photographed using a CCD camera (UVP, UK).

Cleavage-amplified polymorphic sequence (CAPS)

CAPS was generated for the J888 marker using the primer pair 5`-AGACCCAGAGCACACACAGC-3` (forward) and 5`-AGACCCAGAGGATGAGGAATG-3` (reverse) to recover 888 bp as previously described (Ince and Karaca, 2011). The amplicons were shipped to BGI, China for Sanger sequencing and sequences were analyzed via using SnapGene® in order to detect Single Nucleotide Polymorphism (SNP) markers and make predicted restriction analysis towards the detection of possible polymorphic sex-specific bands.

Amplified fragment length polymorphism (AFLP)

AFLP analysis was performed using the two combinations EcoRI-GC/MseI-GCG and EcoRI-TAC/MseI-GCG of the AFLP Analysis System I (Invitrogen, cat. no. 10544-013) following manufacturer's protocol. Genomic DNAs of the five male and five female samples were digested with EcoRI and MseI restriction enzymes in which EcoRI and MseI adapters were ligated to the digested DNA fragments. Pre-amplification was carried out using EcoRI primer plus one extension base at the 3' position (G or T) and MseI primer plus one extension base at the 3' position (G) to amplify fragments that contain complementary sequences.

Whole genome sequencing

Whole genome sequencing was done for one male and one female randomly-selected jojoba samples at BGI, China. About 60 million 100-bp paired-end reads were generated from sequencing libraries with 500-bp insert. Data were filtered to remove the low-quality reads and trim adaptor using Fastp package

(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6129281/). The remaining sequencing reads from the two samples were *de novo* assembled via velvet (v1.2.10) without length cutoff as described (https://www.ebi.ac.uk/~zerbino/velvet/). Contigs recovered from the two sexes were further used in detecting either new or previously-known sex-specific markers in jojoba. The raw data was deposited in the Short Read Archive database (BioProject ID PRJNA603451) of the NCBI.

Detection of new sex-specific markers

Multiple contig alignment of the genomes of the two sexes was done using NUCmer module 3.0 (NUCleotide MUMmer, part of mummer software) to determine the maximal unique matches of a given length (350-500 bp) between the two input sequences, a step to increase the overall coverage of the alignment (Kurtz et al., 2004). A number of 100 contigs of each genome showed high matching in DNA sequences. Contig pairs with high similarity (~90%) and unique sex-specific areas of 350-500 bp (sequence similarity/difference criteria) were selected and sex-specific primers were generated (*Appendix 1*). PCR was performed using ready master mix (BioTaq Green Master Mix, Promega) and conditions were 95 °C/5 min (initial denaturation), 95 °C/30 s, 52 °C/45 s and 72 °C/1 min (40 cycles), 72 °C (final extension), then reaction was held at 4 °C. Amplicons were run on agarose gel, stained with ethidium bromide (0.3 ug/ml), then visually examined and photographed.

Detection of previously-known sex-specific markers

Resulted contigs from both sexes were blasted (ncbi-BLAST v 2.10.0) against previously-known sex-specific sequences referring to an ISSR marker namely UBC-807_{1200} (Sharma et al., 2008; Heikrujam et al., 2014), sequence-related amplified polymorphism (SRAP) (Kumar et al., 2019) determining sex in jojoba as well as *OGI/MeGI* system determining sex in persimmons (*Diospyros* spp.) (https://www.ncbi.nlm.nih.gov/nuccore/KM408640) (Akagi et al., 2014).

Results and discussion

Production of jojoba has spread in many regions of the world (Benzioni et al., 2005) including the Middle East. However, no previous comparative analysis has been made at the molecular genetic level to detect genetic distances among genotypes existing in different regions. Therefore, there is no guarantee that the genetic makeup of the genotypes in Saudi Arabia will harbor the same molecular markers published elsewhere.

In the present study, several types of molecular markers using specific primers were proven not to be useful in determining sex in jojoba plants growing in Saudi Arabia (*Appendix 2*). As models, amplicons of two RAPD (with A02 and B09 primers) and two ISSR (with 544A and HB11 primers) are shown in *Appendix 2*. We speculate one reason for the failure to detect the known sex-specific RAPD or Td-PCR markers is that these two types of markers are likely genotype-specific. Identification of molecular marker for ascertaining sex of plants at seedling stage is vital for optimal plantation of dioecious plants including jojoba. This approach has previously been followed in many plants including *Pistacia vera* (Hormaza et al., 1994), *Carica papaya* (Deputy et al., 2002) and *Mercurialis annua* (Khadka et al., 2002) using random amplified polymorphic DNA (RAPD) approach, while sex in plants like *Asparagus officinalis*

(Reamson-Büttner et al., 1998), *Cannabis sativa* (Flachowsky et al., 2001; Peil et al., 2003) and *Ficus fulva* (Parrish et al., 2004) was determined using amplified fragment length polymorphism (AFLP) approach. Previous studies to detect sex-specific molecular markers in jojoba included a number of RAPD (Agrawal et al., 2007), touchdown polymerase chain reaction (Td-PCR) (Ince et al., 2010) and cleavage-amplified polymorphic sequence (CAPS) (Ince and Karaca, 2011) markers. Out of 72 RAPD primers, only one primer, e.g., OPG-5 (5`-CTGAGACGGA-3`), produced a male-specific marker of ~1.4 kb (Agrawal et al., 2007). Using Td-PCR, another male-specific marker, namely JMS900, was detected using a 10-mer primer (5`-AGACCCAGAG-3`) (Ince et al., 2010). Mohasseb et al. (2009) indicated that one reason for the failure to generate reliable information from these two types of markers is that they might be vulnerable to PCR conditions and quality of the genomic DNA.

The previous results of CAPS with the J888 marker indicated that the 888 bp digested with ClaI, HindIII and HinfI produced polymorphic fragments in male and female samples (Ince and Karaca, 2011). In the present study, this was not the case as digestion of the 888 bp amplicon with these three restriction enzymes generated no polymorphic sex-specific bands (data provided upon request). Therefore, the J888 marker was sequenced for five male and five female samples and multiple sequence alignments of the forward and reverse sequences were done using Clostal Omega (*Appendices 3* and *4,* respectively). The results indicated the presence of two sex-specific SNPs shown in both the forward [sites 138 (G > A) and 330 (C > T)] (*Table 1* and *Appendix 3*) and reverse [sites 609 (C > T) and 416 (G > A)] (*Table 1* and *Appendix 4*) directions of the clean sequences.

Table 1. *List of sex-specific SNPs either lying within restriction sites (colored boxes) or not (clear boxes) across five male (J1-J5) and five female (J6-J10) samples existing in the CAP marker J888. Sanger sequencing was done for J888 amplicon at the two (forward and reverse) directions. SNPs are mainly C/T and A/G, while restriction sites are for Bsp119I and TaqI enzymes. Note that TaqI site (T↓CGA) exists within Bsp119I site (TT↓CGAA). Red = male, blue = female. Multiple sequence alignments of the forward and reverse sequences of J888 marker using Clostal Omega are shown in Appendices 1 and 2, respectively*

Primer	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	SNP position (type)/restriction site
Forward	A	A	A	A	A	A	A	A	A	T	44 (A > T)
	G	G	G	G	G	A	A	A	A	A	138 (G > A)/TaqI
	G	G	G	G	G	G	G	G	G	A	268 (G > A)
	C	C	C	C	C	T	T	T	T	T	330 (C > T)/Bsp119I/TaqI
Reverse	G	G	G	G	G	A	A	A	A	A	416 (G > A)/Bsp119I/TaqI
	C	C	C	C	C	C	C	C	C	T	479 (C > T)
	C	C	C	C	C	T	T	T	T	T	609 (C > T)/TaqI
	T	G	G	G	G	G	G	G	G	G	678 (G > T)
	A	G	G	G	G	G	G	G	G	G	695 (G > A)

In addition, restriction analysis and maps of one randomly selected male (*Appendix 5*) and one selected female (*Appendix 6*) samples were detected using SnapGene®. The latter software predicted the occurrence of several polymorphic sex-specific bands when the amplicon is digested with either restriction enzymes TaqI or

Bsp119I (*Fig. 1* and *Appendix 7*). These polymorphic bands are consequences of the two SNPs sites located within the male and female of the J888 marker sequences. For TaqI, two male-specific markers with 125 and 70 bp, while two female-specific markers with 173 and 90 bp were detected. For Bsp119I, three male-specific markers with 80, 70 and 53 bp, while two female-specific markers with 123 and 90 bp were detected. Note that the TaqI site (T↓CGA) exists within Bsp119I site (TT↓CGAA). The predicted number of restriction fragments of TaqI enzyme is much higher than that of Bsp119I as the recognition site of the first is 4-base, while 6- base for the second. We expected that DNA sequences of the 90 bp female-specific marker across the two restriction enzymes are the same. Then, we claim that we successfully detected two SNPs markers that resulted in the production of five male-specific and four female-specific markers when the J888 amplicon was digested with both TaqI and Bsp119I restriction enzymes.
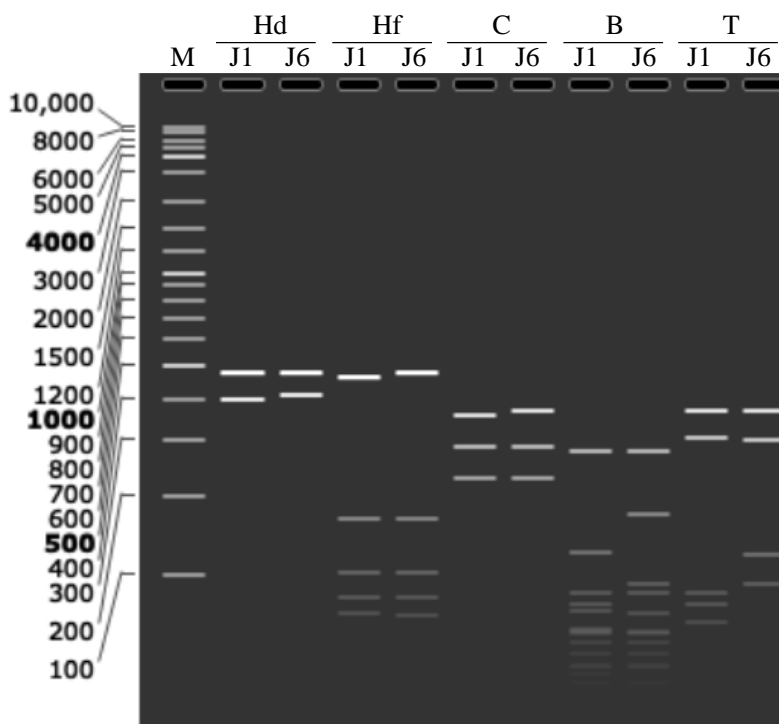

Figure 1. Predicted restriction analysis displayed on a virtual gel for one male (sample J1) and one female (sample J6) samples existing in the CAP marker J888. Restriction enzymes involved HindIII (Hd), HinfI (Hf), ClaI (C), Bsp119I (B) and TaqI (T). Note that TaqI site (T↓CGA) exists within Bsp119I site (TT↓CGAA). M = 100 bp-ladder. Sizes of restriction fragments are shown in Appendix 3. Complete restriction maps of J888 markers for the male and female samples are shown in Appendices 4 and 5. Restriction fragment sizes and maps were detected using SnapGene®

AFLP is a reliable approach in detecting molecular markers in dioecious plants (Mwase et al., 2007), while RAPD, a dominant marker, has several drawbacks and lacks reproducibility (Mohasseb et al., 2009). Then, AFLP has a large chance to detect sex-specific markers in jojoba (Agarwal et al., 2011) as this type of markers allows the testing of large genomic fragments at once compared with the earlier types of molecular markers that test very small regions of the genome. Previous efforts utilizing AFLP in detecting sex-specific markers resulted in the recovery of two male-specific markers of

~525 and 325 bp using primer combinations EcoRI-GC/MseI-GCG and EcoRI-TAC/MseI-GCG, respectively, while only one female-specific marker of ~270 bp using the primer combination EcoRI-TAC/MseI-GCG. In the present study, the primer combination EcoRI-GC/MseI-GCG resulted in the recovery of five (428, 307, 190, 163 and 60 bp) male-specific and three (768, 401 and 25 bp) female-specific markers (*Appendix 8*), while the primer combination EcoRI-TAC/MseI-GCG resulted in the recovery of three (340, 196 and 56 bp) male-specific and three (425, 241 and 179 bp) female-specific markers (*Appendix 9*). We disconsidered sex-specific markers with > 25 bp. Dendrograms utilizing the two combinations resulted in the complete separation of the two sexes (*Figs. 2* and *3*). Then, we claim that this type of marker successfully determines sex in jojoba with high efficiency.
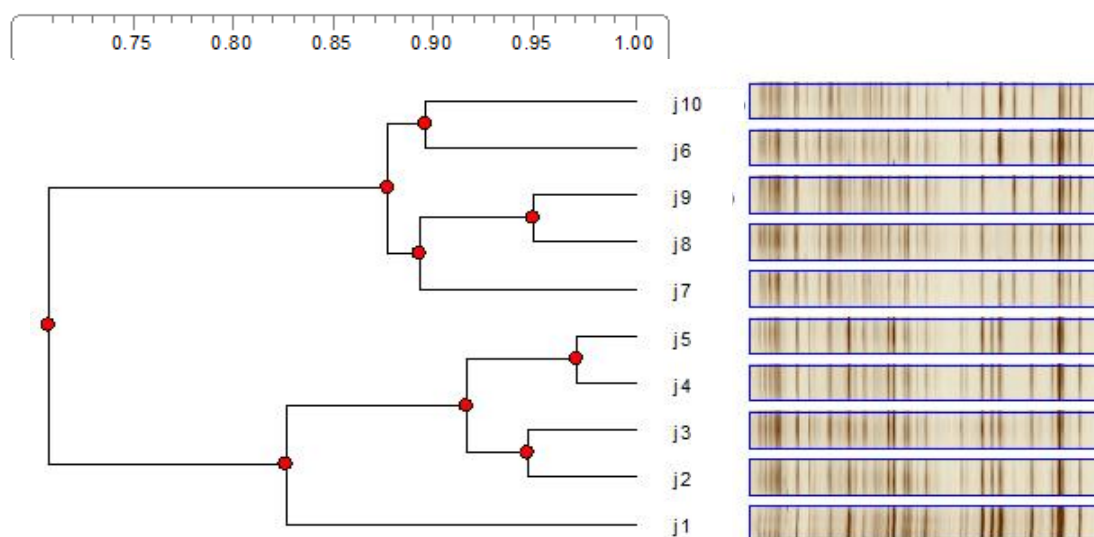
Figure 2. *Dendrogram resulted from AFLP with the primer combination EcoRI-GC/MseI-GCG to describe the relationship between male (J1-J5) and female (J6-J10) banding patterns*
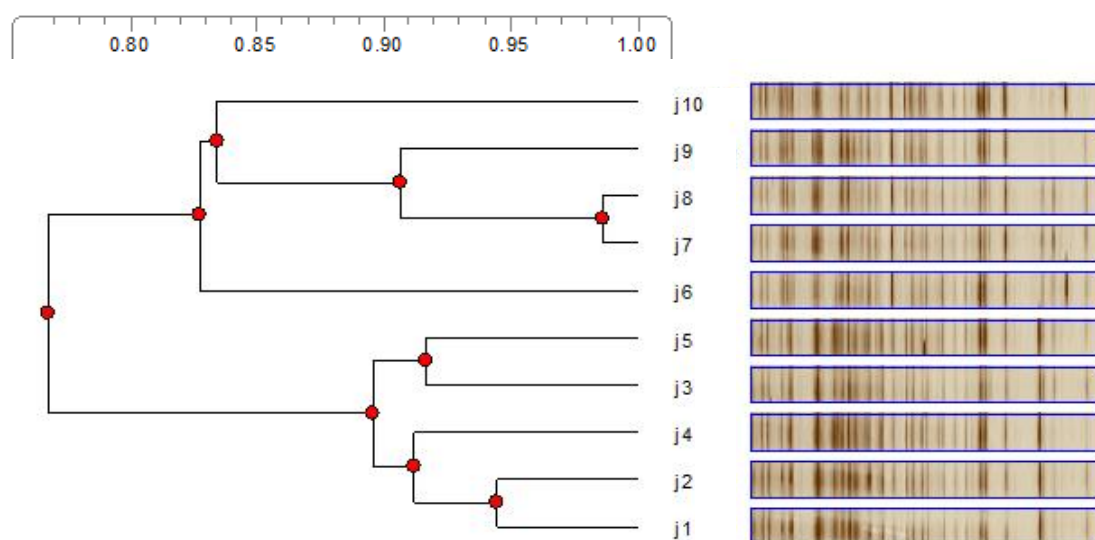
Figure 3. *Dendrogram resulted from AFLP with the primer combination EcoRI-TAC/MseI-GCG to describe the relationship between male (J1-J5) and female (J6-J10) banding patterns*

Multiple contig alignment of the genomes of the two sexes done using NUCmer module 3.0 (NUCleotide MUMmer, part of mummer software) indicated a number of 18 male and 17 female unique sequences of 350-500 bp. PCR with the designed sex-specific primer combinations was done for one male and one female samples and results indicated the occurrence of four male-generated (Rx9, Rx11, Rx26 and Rx33) and two female-generated (Rx1 and Rx4) amplicons (*Appendix 2*). Interestingly, the male-generated amplicons of Rx9 and Rx11 were supposed to be possible female-specific markers. PCR for five male and female samples indicated arbitrary inconsistent presence/absence of the four male and two female-generated amplicons in the two sexes (*Appendix 2*). We speculate that the failure to detect sex-specific markers via multiple contig alignment of the genomes of the two sexes and the use of MUMmer software is due to the need for larger yield or coverage of raw reads of genomes of the two sexes or due to the lack of appropriate binding sites in the template. Low coverage results in the presence/absence of many contig sequences that are not sex-specific. Therefore, we recommend repeating the whole genome sequencing of genomes of the two sexes, but requesting larger read coverage.

Two male-specific ISSR (Sharma et al., 2008; Heikrujam et al., 2014) and sequence-related amplified polymorphism (SRAP) (Kumar et al., 2019) markers have also been validated in the present study. ISSR was previously reported to result in the recovery of a male-specific marker namely UBC-807$_{1200}$ utilizing primer UBC-807, while SRAP was reported to result in the recovery of a male-specific marker with an amplicon size of 396 bp utilizing primer combination Em14/Me10. In the present study, we have sequenced the whole jojoba male and female genomes and blasted the *de novo* assembled contigs of the two genomes against the published sequence of the male-specific ISSR marker in the NCBI (acc. no. HQ166029). The results indicated the presence of several fragments of the marker sequence in both male (*Fig. 4* and *Appendix 10*) and female (*Fig. 4* and *Appendix 11*) genomes. Results of the blasted male and female *de novo* assembled genomes against the published sequence of the SRAP male-specific marker of Kumar et al. (2019) with primer combination Em14/Me10 are shown in *Appendices 12* and *13,* respectively. The results proved that the DNA sequence of the SRAP marker was almost completely detected in the male sample (*Fig. 5*), while completely absent in the female sample. We concluded that the SRAP marker can successfully determine males in jojoba with high efficiency, while the UBC-807$_{1200}$ marker was not proven to be male-specific.

Dioecy is often associated with the occurrence of sex chromosomes in plant and the presence of genetic determinants of sex (Ming et al., 2011; Renner, 2014; Charlesworth, 2016). After the era of genomics and whole genome sequencing (WGS), it is now possible to decipher the architecture of sex chromosomes in several dioecious plants (Liu et al., 2004; Ming et al., 2011; Wang et al., 2012, 2013; Charlesworth, 2016; Kazama et al., 2016; Harkess et al., 2017; Muyle et al., 2017). Genetic determinants of sex were deciphered in some species, including persimmons (*Diospyros* spp.) (Akagi et al., 2014), garden asparagus (*Asparagus officinalis* L.) (Harkess et al., 2017) and kiwifruit (*Actinidia* spp.) (Akagi et al., 2018). Sex was determined in persimmons through the action of one single non-coding RNA gene located on the Y chromosome namely *OGI* gene (Yang et al., 2019). This gene produces a small-RNA sequence that targets an autosomal counterpart gene namely *MeGI* (Yang et al., 2019). The latter is thought to be a single integrator of sex expression (Akagi et al., 2014, 2016; Henry et al., 2018; Yang et al., 2019). As *OGI* gene is located on Y chromosome, we expected

that if this sex determination system exists in jojoba, then existence of *OGI* gene will be a male-specific marker, while we expect that the *MeGI* gene exists in the two sexes. BLAST results of the *de novo* assembled contigs in the present study against the published sequences of the *OGI* and *MeGI* genes are shown for male (*Appendix 14* and *15,* respectively) and female (*Appendix 16* and *17,* respectively) jojoba genomes. The sequences of *OGI* and *MeGI* genes of persimmon are available in the DDBJ database, with the Illumina reads for the mRNA-Seq analysis deposited in the Short Read Archive database (BioProject ID PRJDB7688) of the NCBI. The BLAST results indicated that *OGI* gene was not detected in either jojoba sexes, while *MeGI* gene was detected in 12 and six contigs of the jojoba male and female samples, respectively. Prior information neither confirmed the existence of Y chromosome in male plants of jojoba, nor proved the existence of *OGI*/*MeGI* system in jojoba. Existence of *MeGI*, while lack of *OGI* is a question remains to be answered. In general, we confirmed that the published *OGI*/*MeGI* system does not exist to drive sex in jojoba.
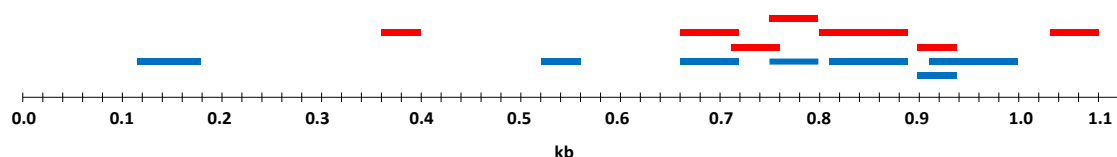
Figure 4. BLAST results for the DNA fragments within the UBC-807$_{1200}$ sequence (acc. no. HQ166029) existed in the male (blue) and female (red) de novo assembled genomes of jojoba growing in Saudi Arabia
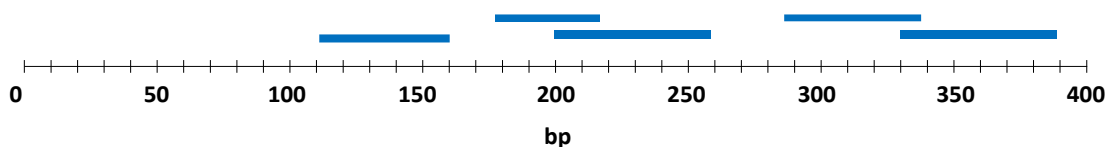
Figure 5. BLAST results for the DNA fragments within the SRAP male-specific marker (396 bp) existed in the male de novo assembled genome of jojoba germplasm growing in Saudi Arabia

Conclusion

Overall results of the present study indicated the possibility to utilize CAPS with either restriction enzymes TaqI or Bsp119I in detecting sex-specific markers in jojoba. Also, AFLP resulted in the recovery of several sex-specific markers, thus, seems to be very useful in separating the two sexes in jojoba very efficiently. The previously described male-specific SRAP marker was also confirmed in jojoba plants growing in Saudi Arabia. These three types of sex-specific markers might be useful in the future industry of biofuel production in Saudi Arabia and the Arab region. The study also refers to the necessity to barcode this important plant in different regions of the country and re-validate the recovered sex-specific markers way before we decide to incorporate this plant in breeding program or at commercial scale.

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ELECTRONIC APPENDIX

Appendix 1. List of RAPD (Operon Technologies, USA) and ISSR primers along with their nucleotide sequences as well as sex-specific primers generated from NUCmer

Appendix 2. Banding patterns randomly selected for RAPD (a) and ISSR (b) analyses as well as those generated from NUCmer (c & d) in a trial to detect new sex-specific markers. As models, amplicons of two RAPD (with A02 and B09 primers) and two ISSR (with 544A and HB11 primers) are shown. (c) Patterns generated from 35 reactions (Rx) of one male and female samples, (d) patterns of five male and five female samples for selected reactions. Mw = 50bp DNA Step ladder, 1-5 = M, 6-10 = female, M = male, F = female. Primer sequences are shown in Appendix 1

Appendix 3. *Forward J888 marker sequences of five male and five female samples and multiple sequence alignments using Clostal Omega*

Appendix 4. *Reverse J888 marker sequences of five male and five female samples and multiple sequence alignments using Clostal Omega*

Appendix 5. *Restriction map of J888 marker for the male sample*

Appendix 6. *Restriction map of J888 marker for the female sample*

Appendix 7. *Virtual gel describing predicted restriction analyses of ClaI, HindIII, HinfI, TaqI and Bsp119I of the 888 bp amplicon for one male and one female samples in jojoba using SnapGene® software*

Appendix 8. *AFLP analysis for the primer combination EcoRI-GC/MseI-GCG in five male and five female samples in jojoba*

Appendix 9. *AFLP analysis for the primer combination EcoRI-TAC/MseI-GCG in five male and five female samples in jojoba*

Appendix 10. *BLAST analysis of the de novo assembled contigs of the jojoba male genome against the ISSR male-specific marker namely UBC-807$_{1200}$ generated by PCR with primer UBC-807*

Appendix 11. *BLAST analysis of the de novo assembled contigs of the jojoba female genome against the ISSR male-specific marker namely UBC-807$_{1200}$ generated by PCR with primer UBC-807*

Appendix 12. *BLAST analysis of the de novo assembled contigs of the jojoba male genome against the SRAP male-specific marker (396 bp) generated by PCR with primer combination Em14/Me10*

Appendix 13. *BLAST analysis of the de novo assembled contigs of the jojoba female genome against the SRAP male-specific marker (396 bp) generated by primer combination Em14/Me10*

Appendix 14. *BLAST results of the de novo assembled contigs of male jojoba plant against the published sequences of the OGI gene*

Appendix 15. *BLAST results of the de novo assembled contigs of male jojoba plant against the published sequences of the MeGI gene*

Appendix 16. *BLAST results of the de novo assembled contigs of female jojoba plant against the published sequences of the OGI gene*

Appendix 17. *BLAST results of the de novo assembled contigs of female jojoba plant against the published sequences of the MeGI gene*