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DETERMINATION OF A SEX-RELATED RAPD MARKER IN CAROB (*Ceratonia siliqua* L.)

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Molecular markers are used in the characterization and breeding of organisms. Carob (*Ceratonia siliqua* L.) is a species with both dioecious and hermaphrodite flower forms. The determination of sex at an early stage of growth in this species, whose juvenility period is long, is important in breeding studies. The objective of this study was to identify the sex-related markers using RAPD method. Ten genotypes were obtained from natural F_1 hybrids between a naturally grown a female and a male carob tree. DNA was extracted from the leaves of 12 carob plants. Using BSA, the female and male bulks were formed from five female and five male plants, respectively, using equal amounts of DNA from each plant. In this study, 130 RAPD primers were tested. That of 21 primers tested showed polymorphisms between male and female bulks. While the fragment of 750 bp from the OPA17 RAPD primer was not detected in the female parent, female bulk, and female F_1 hybrids; it was observed in the male parent and four out of five male F_1 hybrids. This is the first report in the literature that one RAPD marker, namely OPA17-750, related to 80% reliability to male sex in carob was determined.

Keyword: Ceratonia siliqua, gender, molecular markers, BSA, PCR

INTRODUCTION

Carob (*Ceratonia siliqua* L. (2*n*=2*x*=24, Caesalpinoideae) is one of the ancient species among the grapes and olive dated back to Egyptians around the 18th century BCE in the Mediterranean Basin where is considered the center of origin. The Caesalpinoideae subfamily,

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consists of mainly trees distributed in the moist tropics but include such temperate species, is placed in a large family of Leguminosae, syn: Fabaceae. The genus *Ceratonia* has two species, namely *C. siliqua* and *C. oreothauma*. World carob pod production is estimated about 220,000 t per year produced from 127,000 ha. Spain is the leading country followed by Morocco, Italy, Portugal, Greece, Turkey, Cyprus, Algeria, Israel, California, and Australia in carob production. Turkish total production is about 12,000-15,000 t that is collected from isolated trees (354,000) as there are no carob orchards. The production is concentrated along the coast in the Mediterranean (96%) and Aegean (4%) regions. The main producing provinces are Adana, Antalya, Aydın, Izmir, Mersin, and Muğla (SECMEN, 1975; TOUS *et al*., 2013). Carob is a long-lived, sclerophyllous evergreen, shrub or fruit tree preferred by consumers for its health benefits of the polysaccharides, galactomannan, fiber, and gum products besides its landscape design and molasses production. Although it belongs to Leguminosae, it does not nodulate by bacteria, and unable to fix nitrogen. Arbuscular mycorrhizal colonization helps nitrogen uptake. Carob has a strong tap root penetrating in deep soil absorbing necessary water and nutrients. The pod (fruit) is called Saint John's Bread or locust bean in many countries, especially *keciboynuzu*, *harnup*, *kharub*, *kharuv*, *harip*, or *balliboynuz* in Middle East (TOUS *et al*., 2013). There are three types, namely fleshy, Sisam, and wild found in nature (SECMEN, 1975; GUBBUK *et al*., 2010). Carob has dioecious and some hemaphrodite forms. Besides other properties, Arab jewelers in the Middle Ages used the uniform seeds of carob as a unit of weight (200 mg) for gold and precious stones. That unit was called as *karat*. The carob, besides loquat (*Eriobotrya japonica*), is a Mediterranean tree with the main blooming season at the end of the summer and autumn. Insects and wind both play an important role in pollination. The ratio of pistillate to staminate individuals is about 1:1 including a few hermaphrodites. In some places like Morocco, the proportion staminate to pistillate trees in the natural populations is 3:1. Carob trees can be grouped into five floral classes based on sex expression: (1) pistillate, (2) pistillate with occasional perfect flowers (hermaphroditic), (3) perfect with occasional staminate flowers, (4) perfect, and (5) staminate. Even some early season hermaphrodite cultivars show tendency toward pistil development failure later in the season (TOUS *et al*., 2013).

Approximately 5% of the plants species are dioecious within the plantae kingdom. Sex in plants is determined either homomorphic (XX: *Diospyros lotus*, *Vitis vinifera*, *Populus trichocarpa*, *Actinidia chinensis*, *Asparagus officinalis*, ZZ: *Fragaria virginiana*, *Salix viminalis*, UU: *Ceratodon purpureus*) or heteromorphic (XY: *Carica papaya*, *Silene latifolia*, *Rumex hastatulus*, ZW: *Pistacia vera*, UV: *Marcanthia polymorpha*) chromosomes. In the absence of heteromorphic chromosomes, sex is determined by the recombination suppression between of sex-linked regions in the genome (CHARLESWORTH, 2016).

Molecular markers are DNA fragments, used in the characterization and breeding of organisms, associated with any gene region(s) in the genome. BSA is a powerful tool to differentiate individuals having only two opposite characteristics, i.e., female or male (MICHELMORE *et al*., 1991). Besides inexpensive and easy-to-use properties, RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) is used in determination of random genomic regions linked to certain plant characteristics (WELSH and MCCLELLAND, 1990; WILLIAMS *et al*., 1990). RAPD analysis was used with molecular characterization in *Vitex agnus-castus* L.(Verbenaceae) (SEVINDIK *et al*., 2019), genetic diversity in *Sorghum bicolor* (RUIZ-

CHUTÁN *et al*., 2019), and genetic variation and molecular relationships in *Conringia* Heist. ex Fabr. (Brassicaceae) (SEVINDIK *et al*., 2020). Sex-linked RAPD markers were described in *Pistacia* spp. (HORMOZA *et al*., 1994; KAFKAS *et al*., 2001; NOSRATI *et al*., 2012), *A. chinensis* (HARVEY *et al*., 1997), *A. officinalis* (JIANG and SINK, 1997), *Humulus lupulus* (POLLEY *et al*., 1997), *Hippophae rhamnoides* (PERSSON and NYBOM, 1998; ZHOU *et al*., 2018), *Atriplex* spp. (RUAS *et al*., 1998), *Cannabis sativa* (MANDOLINO *et al*., 1999; SAKAMOTO *et al*., 2005), *C. papaya* (DEPUTY *et al*., 2002; MODI *et al*., 2018), *S. viminalis* (GUNTER *et al*., 2003), *Ginkgo biloba* (JIANG *et al*., 2003), *Simmondsia chinensis* (HOSSEINI *et al*., 2011), and *D. lotus* (AKAGI *et al*., 2014)

During literature search, there was not any record encountered on sex inheritance in carob; therefore, this is the first study finding in the respected subject. The objective of this study was to determine sex-related markers using RAPD markers in juvenile phase in carob.

MATERIALS AND METHODS

The plant material of this study is collected from an orchard located at 37°29’29”K, 27°13’31”D in Akköy, Didim, Aydın, Turkey (Figure 1). An F_1 of seedling carob population composed of a single natural cross between a female and a male parent trees at the corner in the orchard. The seeds were obtained from this natural cross and sown by the owner of the orchard. From this F_1 progeny, five female (F1-5) and five male (M1-5) 8-year-old plants along with these respected female (F) and male (M) parents were used in the study.

Figure 1. Carob plants used in this study A: Female plant, B: Male plant, C: Hybrid plants

DNA isolation using young leaves was made according to modified 2× CTAB protocol (COSTA and ROBERTS, 2014). The quality of DNA was measured by 1% TBE (Tris Borate EDTA) agarose gel and spectrophotometer (NanoDrop 2000 Thermo Fisher Scientific, Waltham, Massachusetts, USA). The DNA amount is equalized to 50 ng/µl for RAPD-PCR analysis. For BSA, an equal amount of DNA from five female (F1, F2, F3, F4, F5) and five male (M1, M2, M3, M4, M5) plants were combined in 1.5 ml Eppendorf tubes (MICHELMORE *et al*., 1991). PCR condition was modified from DALKILIÇ *et al*. (2011) and ORUÇ and DALKILIÇ (2017) as follows: 1.5 µl 10× Buffer, 0.6 µl 25 mM $MgSO_4$, 1 µl 3 mM dNTP, 1 µl primer, 0.2 µl *Taq* DNA polymerase, 4 µl genomic DNA, and 6.7 µl steril ddH_2O in total 15 µl. OPA, OPB, OPC, OPD, OPH, and OPM 120 RAPD primers (Operon Technology (http://www.operon.com.tr) and Z custom synthesized primers were used. PCR program modified from (DALKILIÇ *et al*., 2011) and ORUÇ and DALKILIÇ (2017) was at 94°C for 4 min initial denaturation followed by at 94°C for 25 s denaturation, at 35°C for 45 s annealing, and at 72°C for 1 min elongation for 34 cycles, and at 72°C for 5 min final extension. PCR products were visualized using 1.8% TBE agorose gel running at 90 V for 40 min. SafeView® (ABM, Canada) was used to stain the DNA photographed using UV light source and a camera (Infinity VX2, Vilber Lourmat, BP-66-Z1 Sud Torsy F-77202 Marne-la-Vallée cedex 1, France). Bands on the gel were recorded as present (1) and absent (0).

RESULTS AND DISCUSSION

The DNA content and purity of 260 nm/ 280 nm were found between 77.7 ng/µl and 1.79 (M) and 706.9 ng/µl and 1.96 (M5), respectively. In the first screen using a female bulk and a male bulk, 130 RAPD primers gave 560 total bands. From these, 34 (6.1%) was polymorphic. In the second screening, 21 RAPD primers showed polymorphism between female and male bulks. Of these, six primers showed female-linked bands (300-1700 bp) and eight primers showed male-linked bands (300-1500 bp) (Table 1). In the third screening, when 21 RAPD primers tested again, 20 of them were lost their polymorphism. Only OPA17 primer (5'-GACCGCTTGT-3') continued its polymorphism. While a band of 750 bp was present in all, but one of the F_1 male progeny and a male parent, it was absent all the F_1 female progeny and a female parent (Figure 2). Therefore, OPA17-750 marker was decided to be a male-linked RAPD marker with 80% reliability. This band was consistently present in six repeated experiments.

In Czech Republic, 46 *Sorghum bicolor* genotypes were assessed for genetic diversity using 15 RAPD primers. From the obtained bands ranged from 200 to 2000 bp, 126 of them were scorable and polymorphism ratio was 89% (RUIZ-CHUTÁN *et al*., 2019). Using seven RAPD primers, 36 plant characteristics in *Vitex agnus-castus* populations were investigated. The closest genetic distances were between Çine pink flower and Çakmar purple flower, and Çakmar pink flower and Çakmar purple flower populations with a value of 0.05556. RAPD markers are useful tools for determining genetic relationships among *Vitex agnus-castus* genotypes (SEVINDIK *et al*., 2019). Phylogenetic analysis were performed using seven RAPD primers in 13 populations of 6 species belonging to *Conringia* spp. populations. RAPD markers found to be useful to determine the genetic relationships between populations of the *Conringia* species (SEVINDIK *et al*., 2020).

Table 1. Base differences between female and male bulks using RAPD-PCR

Primer	Total band no.	Polymorphic band no.	Band size (bp)	
			Female	Male
OPA3	5	2	300, 900	-
OPA17	5	1	-	750
OPA18	3	1	700	-
OPB2	8	1	1700	-
OPB4	6	1	-	500
OPB5	5	1	-	800
OPB10	6	1	600	-
OPC2	10	2	-	480, 2400
OPC4	8	4	-	300, 600, 900, 1450
OPC5	8	2	-	550, 1250
OPC7	5	2	-	300, 400
OPC8	7	1	-	650
OPC9	4	2	-	500, 1500
OPC11	8	2	-	500, 600
OPD13	8	2	-	300, 1500
OPD20	8	3	-	500, 600, 700
OPM8	3	1	-	1000
OPM10	5	1	900	-
OPM18	5	2	700, 900	-
Z1	2	1	-	1200
Z5	6	2	-	1300, 1500

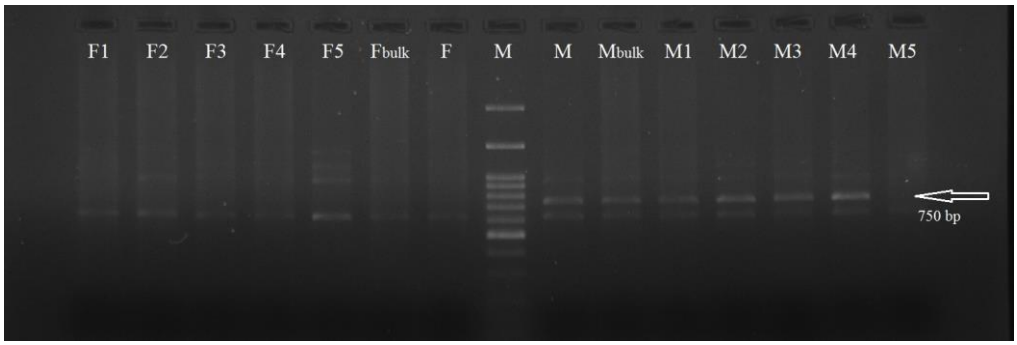

Figure 2. Gel view of OPA17 primer. F1-F5: F_1 female progeny, F_{bulk}: female bulk, F: female parent, M: 100 bp DNA standard, M_{bulk}: male bulk, M1-M5: F_1 male progeny

Plant kingdom contains only about 5% dioecious species (CHARLESWORTH, 2016). Although it has few cultivars with hermaphrodite flowers, carob is an example of an evergreen, dioecious plant having long juvenile period. Since there has not been known sex-related marker by any means in carob, any kind of marker will be very helpful to distinguish individuals according to their sex in their earliest age possible. In the current study, OPA17-750 RAPD band was found 80% associated with a male sex for the first time. OPAK09-850 bp RAPD marker with 100% association in *P. atlantica* and BC360-500 bp RAPD marker with 83% association in *P. eurycarpa* with female sex were reported (KAFKAS *et al*., 2001). RAPD markers of BC210-438 bp linked to with hermaphrodite sex in *C. papaya* (LEMOS *et al.*, 2002), that of $S1478_{682}$ bp linked to male sex in *G. biloba* (JIANG *et al*., 2003), and that of D15-850 bp 100% linked to female in *H. rhamnoides* (ZHOU *et al*., 2018) were found. In the future, more markers need to be discovered with different marker systems higher association with either female or male sex, even hermaphroditism, in carob. Finding more markers will help to saturate genome map in carob.

CONCLUSIONS

This is the first report in the literature that one RAPD marker, namely OPA17-750, related to 80% reliability to male sex in carob was determined. During the juvenile stage of carob, OPA17-750 RAPD marker can be used to differentiate plants either female or male.

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UTVRĐIVANJE POLNIH RAPD MARKERA KOD ROGAČA (*Ceratonia siliqua* L.)

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Izvod

Molekularni markeri se koriste u karakterizaciji i oplemenjivanju organizama. Rogač (*Ceratonia silikua* L.) je vrsta sa dvodomnim i hermafroditskim oblicima cveta. Određivanje pola u ranoj fazi rasta ove vrste, čiji je period maloletstva dug, važno je u uzgojnim studijama. Cilj ove studije bio je identifikovanje polnih markera pomoću RAPD metode. Deset genotipova je dobijeno od prirodnih F1 hibrida između prirodno gajenih ženkih i muških biljaka rogača. DNK je izolovana iz lišća 12 biljaka rogača. Koristeći BSA, ženska i muška masa su formirane od pet ženskih, odnosno pet muških biljaka, koristeći jednake količine DNK iz svake biljke. U ovoj studiji je testirano 130 RAPD prajmera. Ova grupa od 21 testiranog prajmera pokazala je polimorfizam između muške i ženske grupnog uzorka, dok fragment od 750 bp iz prajmera OPA17 RAPD nije otkriven kod ženskog roditelja, ženskog grupng uzorka i ženskih F1 hibrida; primećeno je kod muškog roditelja i četiri od pet muških F1 hibrida. Ovo je prvi primer u literaturi da je utvrđen jedan RAPD marker, naime OPA17-750, koji se odnosi na 80% pouzdanosti muškog pola kod rogača.

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