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DEVELOPMENT OF A RAPD-BASED MALE-SPECIFIC MOLECULAR MARKER IN JAPANESE HOP (*HUMULUS JAPONICUS* SIEBOLD & ZUCC.)

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The male-specific DNA markers are very useful in molecular sexing of non-flowering plants and seeds of dioecious species. In this paper we identified ten Y chromosome-specific RAPD primers suitable for identification of male plants in three Cannabaceae species with sex chromosomes (*Humulus lupulus*, XX/XY; *H. japonicus*, XX/XY1Y2; *Cannabis sativa*, XX/XY). Basing on the nucleotide sequence of the OPJ-09 RAPD product we developed the HJY09 SCAR marker, which is very efficient in sexing of Japanese hop.

Keywords: Cannabaceae, *Humulus japonicus*, SCAR marker, plant sexing

INTRODUCTION

The Japanese hop (*Humulus japonicus* Siebold & Zucc.) is an annual species of the small family Cannabaceae, which is the only angiosperm family consisting exclusively of dioecious species with heteromorphic sex chromosomes. Unlike its closest relative, the common hop (*Humulus lupulus*), the Japanese hop is not useful for brewing industry, due to lack of lupulin glands. In recent years, extracts from *H. japonicus* have been proven to possess various antioxidative, anti-aging, antibacterial, antimutagenic, anti-inflammatory and antitumor properties (Sung et al., 2015).

H. japonicus is characterized by a multiple (polymorphic) sex chromosome system (2n=16, XX in females and 2n=17, XY1Y2 in males) (Sinoto, 1929; Winge, 1929) which distinguishes it from two other Cannabaceae species, the common hop (*H. lupulus*) and hemp (*Cannabis sativa*), both possessing 2n=20 chromosomes and the simple XX/XY chromosome system (Vyskot and Hobza, 2004). The lower chromosome count and different sex chromosome system suggest that *H. japonicus* has derived and highly reorganized karyotype with a sex chromosome system originating from the XX/XY one. Regardless of the sex chromosome system, sex determination in Cannabaceae relies on X-to-autosome balance (X/A≥1.0 in females and X/A≤0.5 in males) (Ainsworth, 2000). In terms of both sex chromosome and sex determination systems, *H. japonicus* resembles *Rumex acetosa*, a model species in sex chromosome research in plants (Navajas-Perez, 2012). Despite the clear karyotypic difference between them, both hop species showed almost identical cpDNA organization. Moreover, comparative analysis of nuclear rDNA coding regions in *H. lupulus*, *H. japonicus* and *C. sativa* revealed high similarity of Cannabaceae species in this respect (Pillay and Kenny, 2006).

The two species with a simple sex chromosome system (*C. sativa* and *H. lupulus*) differ drastically in the size of the Y chromosome. Moreover, *H. lupulus* is the only angiosperm species with the Y much smaller than X. It may arouse suspicion that sex chromosomes observed in different Cannabaceae

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species might not show common origin. In animals there is a well-known and frequently described phenomenon called sex chromosome turnover, which causes that the ancestral sex chromosomes are not conserved during evolution even in closely related species. Sex chromosome turnover is particularly probable in organisms possessing the sex determination system X:A, e.g. in *Drosophila* (Carvalho and Clark, 2005). Because Y chromosomes in both *Humulus* species demonstrate significant differences (Y chromosome in *H. lupulus* is completely euchromatic and small, whereas Y chromosomes in *H. japonicus* are big and heterochromatinized), their homology requires investigation.

In comparison to *H. lupulus* and *C. sativa*, which are commercially important species, genetic studies on *H. japonicus* were neglected for a long time. One of the obstacles in genetic research on the Japanese hop is the paucity of molecular markers suitable for studies on the karyotype evolution in this species and useful in identification of sex chromosomes and in molecular sexing of non-flowering plants and seeds. In the last years, however, some progress has been made, mainly in the knowledge of the karyotype structure and genome size in *H. japonicus* (Grabowska-Joachimiak et al., 2006, 2011; Kim et al., 2008; Alexandrov et al., 2012). More recently, a sex chromosome-specific DNA library has been constructed in this species with the use of laser microdissection (Yakovin et al., 2014).

Grabowska-Joachimiak et al. (2011) suggested that the chromosome complement of *H. japonicus* could have arisen by the X-autosome plus autosome-autosome fusions. Such events have led to the emergence of second Y chromosome and reduction in the autosome number from 18 to 14. The autosome fusion was confirmed by interstitial telomeric sequences located in the large autosome pair, but molecular traces of the X-autosome fusion were not found. Thus, the origin of *H. japonicus* XX/XY1Y2 sex chromosome system from this presented in *H. lupulus* still needs confirmation. A good way to shed light on this question could be search for male-specific (Y-specific) DNA sequences occurring in both species. The X-autosome fusion retains an original Y in the derived sex chromosome system, and some DNA markers located on this chromosome should be observed in both species.

To date, only two Y chromosome-markers have been developed in the Japanese hop. Aleksandrov et al. (2011) described K-16, a male specific ISSR (Inter Simple Sequence Repeat) marker, while Gao et al. (2010) developed a male specific SCAR (Sequence Characterized Amplified Region) marker SEX I64 which was based on ISSR marker I64. According to our findings, both markers were ineffective in *H. lupulus* (Mizia, unpublished results). In this study, we performed RAPD analysis of *H. japonicus* plants in order to develop other male-specific markers useful in sexing of plants and future studies of Y chromosomes in hops.

MATERIAL AND METHODS

PLANT MATERIAL

Molecular analysis of *H. japonicus* included 31 flowering plants (16 males and 15 females) grown from purchased seeds (W. Legutko plant breeders, Poland). Karyological analysis showed that chromosome constitution of all these plants was consistent with their sex phenotype (males 2n=17 and females 2n=16). The usefulness of the developed HJY09 SCAR primers was tested on seeds from Vilmorin Garden plant breeders (France) and PlantiCo plant breeders (Poland). The analyzed *Humulus lupulus* plants (5 males and 5 females) originated from a wild population in Rękawka (Kraków, Poland). The examined *Cannabis sativa* plants (5 males and 5 females) were obtained from commercially available seeds (PPUH Bratek, Poland).

DNA ISOLATION

DNA was isolated from fresh leaves using CTAB procedure (Gavel and Jarret, 1991) with the modification described in Mizia et al. (2014). DNA from seeds was isolated using Gene MATRIX Food-Extract DNA purification Kit (EURx Sp. z o.o. Gdansk, Poland).

RAPD REACTIONS

Thirty RAPD primers were pre-tested on five male and five female *H. japonicus* plants (Tab. 1). Three of them (OPA-07, OPJ-09 and OPU-08) were previously described by Polley et al. (1997) as producing male-specific DNA fragments in *H. lupulus*, whereas two others (RAPD 8 and RAPD 11) were described as producing male-specific DNA fragments in *C. sativa* (Sakamoto et al., 1995). RAPD reaction mixture and amplification were made as described in Mizia et al. (2014). Only primers generating reproducible band profiles were used in further research.

DNA FRAGMENT ISOLATION, CLONING AND SEQUENCING

Male specific bands were extracted from 1% agarose gel with 1X TBE using GenMatrix Agarose-Out DNA Purification Kit (EURx Sp. z o.o. Gdansk, Poland). The purified products were then ligated with an *E. coli* plasmid (Green and Sambrook, 2012). Ligation was performed using CloneJET PCR Cloning Kit (Thermo Scientific). Plasmids purifications were performed using GeneMATRIX Plasmid

TABLE 1. Nucleotide sequences of tested random primers; RAPD – Sakamoto et al., 1995, OPA-04 – OPU-08 – Operon Technologies Inc.

Primer	Sequence (5'–3')	Primer	Sequence (5'–3')
RAPD1*	GCAAGTAGCT	OPA-04*	AATCGGGCTG
RAPD2*	TGGCTCAAAG	OPA-07*	GAAACGGGTG
RAPD3*	CAGTGTGTGG	OPA-17	GACCGCTTGT
RAPD4*	GTGTCAGGCA	OPB-07	GGTGACGCAG
RAPD5*	ATACCATCCC	OPC-01*	TTCGAGCCAG
RAPD6	GATCCCCTGA	OPC-10	TGTCTGGGTG
RAPD7*	GATAACCGCA	OPD-12	CACCGTATCC
RAPD8*	ATCCGCGTTC	OPD-17	TTTCCCACGG
RAPD9*	CCAGTGGTTC	OPE-05	TCAGGGAGGT
RAPD10*	TGACGATGCA	OPF-11	TTGGTACCCC
RAPD11*	ACGGCATATG	OPG-20	TCTCCCTCAG
RAPD12*	TAACCATCCC	OPH-14	ACCAGGTTGG
RAPD13*	ATGTCCGCAC	OPI-03	CAGAAGCCCA
RAPD14*	GTGTGGATGG	OPJ-09*	TGAGCCTCAC
RAPD15	GTCGTTACGA	OPU-08*	GGCGAAGGTT

* – used in further investigation

Miniprep DNA Purification Kit (EURx Sp. z o.o. Gdansk, Poland). The purified plasmids were then sent to Genomed S.A. (Warsaw, Poland) for sequencing. SCAR primers were designed based on the obtained sequences. All male-specific sequences were uploaded into GenBank and received accession numbers (KX688586- KX688595).

SCAR REACTION MIXTURE AND AMPLIFICATION

PCR mixture for HJY09 and SCAR OPJ-09 (*H. lupulus* from Polley et al., 1997) consisted of (15 μl total) 1X Thermo Scientific DreamTaq Green Buffer (including 20 mM $MgCl_2$); 0.25 mM dNTPs; 0.25 μM of each primer; 1.13 U DreamTaq DNA Polymerase (Thermo Scientific) and 60 ng of DNA and was run in Bio-Rad T100 Thermal Cycler. Amplifications were done according to the following program: initial denaturation step at 94°C for 4 min.; 30 cycles consisting of denaturation step at 94°C for 1 min., primer annealing step at 60°C for 45 s and primer extending step at 72°C for 1 min 30 s; and final extending step at 72°C for 8 min. The PCR products were separated in 1% agarose gel with 1X TBE and SimplySafe (EURx Sp. z o.o. Gdansk, Poland). PCR reaction mixture and amplification for SEX I64 were carried in Bio-Rad T100 Thermal Cycler according to the instructions in Gao et al. (2010).

ANALYSIS OF OBTAINED SEQUENCES

Sequences of both OPJ-09 male-specific products obtained from *H. japonicus* were compared with sequence of male-specific product generated by OPJ-09 for *H. lupulus* by using MultAlin program (Corpet, 1988). Each comparison was made using default settings (best math) and with a gap penalty at opening and extension. High penalty allowed stretching sequences from forward to reverse primer (Fig. S1, supplementary material).

H. japonicus OPJ-09 700 bp sequence was used in nucleotide BLAST search (Camacho et al., 2009) on megablast and discontiguous megablast settings.

RESULTS AND DISCUSSION

The development of sex-chromosome markers can be beneficial in obtaining information useful in basic research on sex determination and sex-specific genome regions (Charlesworth, 2008). Such

markers, located mainly on Y chromosomes, enabled progress in research on sex chromosomes of several dioecious species, including two model species, *Silene latifolia* (Kazama and Matsunaga, 2008) and *Rumex acetosa* (Mariotti et al., 2009; Navajas-Perez, 2012), as well as commercially important *Cannabis sativa* (Sakamoto et al., 1995), *Humulus lupulus* (Divashuk et al., 2011), *Carica papaya* (Liu et al., 2004) and *Asparagus officinalis* (Gao et al., 2007). They were also used for sexing of unflowering plants or seeds (Polley et al., 1997; Zhang et al., 1998; Korpelainen, 2002; Danilova and Karlov, 2006; Gangopadhyay et al., 2007; Hobza and Widmer, 2008; Kwolek and Joachimiak, 2011). The PCR-based DNA fingerprinting techniques commonly used in the development of sex-linked markers in plants include, among others, RAPD (Random Amplified Polymorphic DNA), in which short (usually 10 bp) arbitrary primers are used to generate random genomic fragments, as well as ISSR (Inter Simple Sequence Repeat), which is based on amplification of genome regions between neighboring and inversely oriented microsatellites. The obtained DNA fragments are sometimes used to generate sequence specific markers (STS, SCAR) which are more advantageous (Heikrujam et al., 2015), because they show high reproducibility and are locus-specific.

From the 30 RAPD primers tested in this research, only 18 yielded reproducible band profiles in *H. japonicus* plants. Ten out of these primers also generated male-specific DNA fragments in particular Cannabaceae species (Tab. 2). Only two primers (OPU-08 and OPJ-09) produced male-specific bands both in *H. japonicus* and *H. lupulus*. The male-specific primer common for *Humulus* and *Cannabis* was not found. RAPD profiles produced by OPJ-09 primer showed one male-spe-

TABLE 2. RAPD primers producing male-specific products (MSP) in *Humulus japonicus*, *H. lupulus* and *Cannabis sativa*

Primer	***H. japonicus***		***H. lupulus***		***C. sativa***	
	Number of MSP	Length of MSP [bp]	Number of MSP	Length of MSP [bp]	Number of MSP	Length of MSP [bp]
OPA-07	0	–	1	1700#	0	–
OPC-01	1	450* (KX688586)	0	–	NT	–
OPJ-09	2	1600* (KX688590) 700* (KX688591)	1	1200#* (KX688593)	0	–
OPU-08	1	900* (KX688592)	1	1400#	0	–
RAPD 2	1	2000* (KX688594 – partial KX688595 – partial)	0	–	0	–
RAPD 3	1	300* (KX688587)	0	–	0	–
RAPD 5	2	1200 1000* (KX688589)	0	–	0	–
RAPD 8	0	–	NT	–	1	500$
RAPD 11	0	–	NT	–	1	730$
RAPD 13	3	1100 800* (KX688588) 900	0	–	0	–

* – sequenced by us; # – sequenced by Pooley et al. (1997); $ – sequenced by Sakamoto et al. (1995); grey – products used to obtain efficient male-specific STS/SCAR markers. NT –not tested

cific fragment in *H. lupulus* (~1.2 kb) and two in *H. japonicus* (~1.6 kb and ~700 bp) (Fig. 1). OPU-08 generated one male-specific product both in *H. lupulus* and *H. japonicus*. The product obtained in *H. japonicus* (~1 kb) was smaller than the one obtained in *H. lupulus* (~1.4 kb).

Eight out of eleven male-specific *H. japonicus* RAPD products were cloned and sequenced, and respective SCAR primers were designed. Testing reactions with these primers showed that only one marker (HJY09, based on the shorter OPJ-09 fragment) was highly male-specific in *H. japonicus* plants. The starting OPJ-09 sequence used for the generation of HJY09 marker is given in the Fig. 3. The suitability of our SCAR marker in sexing of *H. japonicus* was also tested on DNA isolated from seeds. The efficiency of this marker in identification of male seeds was confirmed by using the Y-specific SEX I64 marker developed by Gao et al. (2010) (Fig. 2). Unfortunately, HJY09 primers designed by us (5'-TCACCACTTTGAACTCGCTG-3' and 5'-CCTTGTCTGGGTCGATTTGT-3') proved to be ineffective in *H. lupulus* males (data not shown). BLAST analysis of OPJ09 700 bp product did not shown any significant similarity to any sequence stored in database.

In angiosperms, the occurrence of the multiple XX/XY1Y2 sex chromosome system was documented only in some species of *Rumex* (representatives of European section *Acetosa* and in American *R. hastatulus*) and in *H. japonicus* (Mariotti et al., 2009; Grabowska-Joachimiak et al., 2011, 2015). According to the most common hypothesis, the polymorphic sex chromosome system derives from the simple ones (XY) by fusion between an autosome and a sex chromosome (Ohno, 1967; White, 1973). If so, the derived XX/XY1Y2 system of *H. japonicus* should possess both an ancestral and a neo-Y chromosome (of autosomal provenience). The occurrence of male-specific markers amplified by the same primers in the two *Humulus* species could confirm this supposition.

In this study we focused primarily on finding reproducible, sequence-specific sex markers

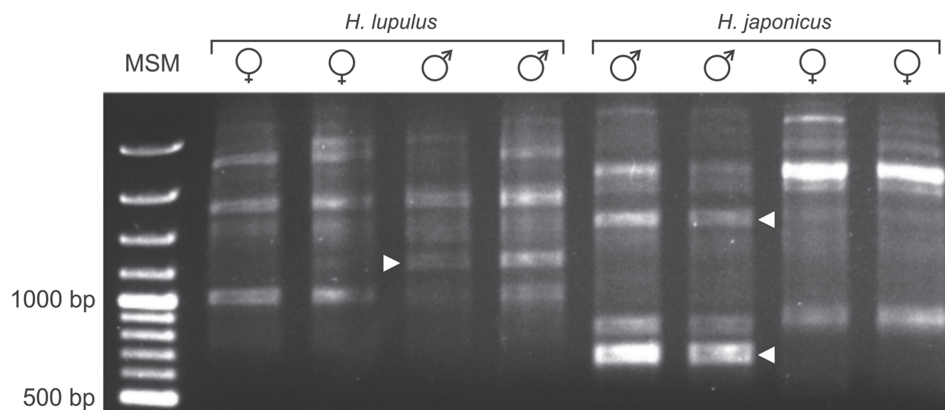

Fig. 1. RAPD profile obtained using OPJ-09 RAPD primer in *Humulus lupulus* and *H. japonicus.* MSM – molecular size marker (100 bp DNA ladder plus – Thermo Scientific); Male-specific fragments marked by arrowheads.

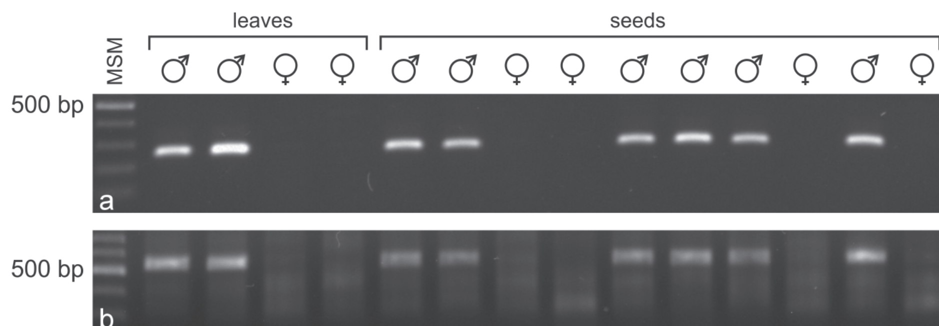

Fig. 2. DNA fragments produced for *Humulus japonicus* plants and seeds by two male-specific markers based on OPJ-09 product: (**a**) HJY09, (**b**) SEX I64. MSM – molecular size marker (100 bp DNA ladder plus – Thermo Scientific).

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TGAGCCTCACCACTTTGAACTCGCTGAATGTTATTAATTGCTGTCCCATGTTATTGAAGA
GATGCAAAAGGGAAATAGGTGAAGATTGGGATAAGATTTCTCATATACAACACTTACTCT
TACGTCCAGATCCCAACGAAAAAGAGGATGAGGTAGAAAATTTAACTTAATCATTTTATT
TTTATTAAATATAACCTAAGTGCTCAGTTTGATTTAGTTTAATTTATTTAACACTGCAGAAT
GCGTTGGAAGAGGAAACAAATCGACCCAGACAAGGGGATGCAACCTCTTGTTGAACAA
GTTTAGGGTATGCAATATTATGAGTACAAGAATTCCAAAATAGATATGTGCAGGTAATTA
TCCTGCTAATCCCTTCTACCTGTATAAACTTCATTCTAATATGATTTAAATGACAAATCTC
AATCTAAAATAAGAACACTTTTCAGCCATTTGAAAGAAAAATATATTTTATCTGTTCAATTA
AAAATCTTGAGCTTATGATTCGTTTGTAAGAACTTCTTACTTTTCTTTTGAGTTCTTGAAA
GACTAAAGCATATCAAAATCCCATTTTGTGGTGATAACATGTTGGTAATATAGGGGGCAT
GCTATGGGTTGAAATTACATTAATGTTCCACTTGGGACTGACTGGTCTGCTACACCTGGT
TATAAAATCTGGTCATCGAGTGAGGCTC
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Fig. 3. Nucleotide sequence of smaller male-specific product generated by OPJ-09 (692 bp). Underlined – the sites of designed HJY09 SCAR primers; Grey – 270 bp fragment produced by designed primers.

in *H. japonicus*, but we also aimed to find RAPD primers effective in generating Y-specific DNA fragments in two *Humulus* species. We found two RAPD primers (OPJ-09 and OPU-08) producing Y-specific bands both in *H. japonicus* and *H. lupulus*. The same primers, tested by Törjék et al. (2002) in *Cannabis sativa*, gave no sex-specific products in this species. 1200 bp DNA fragment amplified by OPJ-09 in *H. lupulus* was used by Polley et al. (1997) to design the Y-specific STS primers for this species. We tested these primers (5'-ACAGAGTACAACTCAGAAACAAACC-3' and 5'-AAGGTCGCACAATGACCG-3') in *H. japonicus*, but they did not amplify any DNA fragments, neither in males nor in females (data not shown). Because the nucleotide sequence of OPJ-09 product in *H. lupulus* has not been published by authors, we cloned and sequenced this DNA fragment. It turned out that the similarity of the OPJ-09 products generated in two *Humulus* species was too small to conclude their common origin (see Fig. S1 in supplementary material). Thus, the occurrence of common OPJ-09 RAPD markers does not prove that *H. japonicus* sex chromosome system originated from the X-autosome fusion in the common ancestor of the two analyzed species. Further molecular-cytogenetic studies with the use of the Y-specific DNA sequences should clarify this issue.

AUTHORS' CONTRIBUTION

AJJ and AG-J plant material, original idea, study design, karyological analysis and critical revision of manuscript; DK, PM, AC-G, MC, MD acquisition of data; DK, PM analysis and interpretation of data; AC-G and PM drafting of manuscript. The authors declare that there are no conflicts of interests.

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