Identification of sex in F_1 progenies of hop (*Humulus lupulus* L.) by molecular marker

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ABSTRACT

Sex identification for hop (*Humulus lupulus* L.), which is a dioecious plant, is very important for breeding process. The use of molecular methods for marker-assisted selection (MAS) provides rapid and reliable identification of sex in F_1 progenies. In the first, we proved the use of specific PCR molecular marker for evaluation of sex in selected plants. This marker was not amplified in 3 from 35 male plants. In the next, we successfully analysed 770 genotypes of F_1 progenies of three crossings. The amplified differences were found in 4 male plants of crossing Sm01 H28, 8 male plants of crossing Sm01 H29 and 5 female plants of crossing Sm00 H20. Statistic analysis confirmed that sex ratios of all F_1 progenies were significantly coincident. Obtained results confirmed that this specific PCR marker successfully identified sex of juvenile hop plants in F_1 progenies and effectively improved breeding process.

Keywords: PCR; marker-assisted selection (MAS)

Hop (*Humulus lupulus* L.) is a dioecious perennial climbing plant without tendrils, bines twining round support in a clockwise direction. Only female inflorescences, referred as cones, are the commercial product of the hop plant (Neve 1991). Genetically, cultivated European hop is diploid, with $2n = 20$ chromosomes of which 9 pairs are autosomal bivalents. Female plants usually have two sex chromosomes of the XX type. Male plants have the same number of chromosomes, including one X and one Y chromosome. It has been suggested that the expression of the male sex is determined by an X:autosome ratio (Parker and Clark 1991, Dellaporta and Calderon-Urrea 1993), although the Y chromosome is indispensable for the full expression of the male phenotype. Because of this, the sex of hop plants can only be reliably determined at the time of flowering, which is 1–2 years after the planting of seeds from a cross. For breeding and selection of new hop hybrid genotypes, it would be highly desirable to have a method for rapidly identifying of female plants at the seedling stage, so that male and female plants could be separated at the time. The use of cytological analysis of X and Y chromosomes is possible only in some hop varieties (Haunold 1991). An use of molecular methods for marker-assisted selection (MAS) in large numbers of plants provides a means for rapid and reliable identification of agricultural important traits in F_1 progenies. The specific molecular markers for sex identification were derived in some dioecious plants: hemp (Sakamoto et al. 1995), pistachio (Hormanza et al. 1994), papaya (Parasnis et al. 2000), *Actinidia* (Gill et al. 2000), *Asparagus* (Reamon-Bűttner et al. 1998) and *Silene* (Zhang et al. 1998). Polley et al. (1997) also developed specific PCR molecular marker associated with Y chromosome of hop.

In our work, we have proved the use of specific PCR molecular marker for evaluation of hop seedlings in F_1 progenies during three years of breeding processes. We have evaluated the usefulness of this molecular marker as male-specific marker in different hop materials and crosses.

MATERIAL AND METHODS

Plant material and DNA isolation

Original plant material was collected from the genetic resources of Hop Research Institute in Žatec and model crossings were carried out during three years. In the first year, 18 genotypes of F_1 progeny Sm99 H30 (Target × 82/6), 37 genotypes of F_1 progeny Sm99 H35 (Magnum × 86/4), 18 genotypes of F_1 progeny Sm99 H39 (Yeoman × 87/3) and 35 genotypes of F_1 progeny Sm99 H45 (Premiant × 87/3) were used for experiments. In the second year, 210 genotypes of F_1 progeny Sm00 H20 (Agnus × Sm98 H35) were used for experiments. In the third year, 170 genotypes of F_1 progeny Sm01 H28 (4706 × O.P.) and 340 genotypes of F_1 progeny Sm01 H29 (4712 × O.P.) were used for experiments. DNA was isolated from young leaves, collected at the beginning of May, by Saghai-Maroof et al. (1984) modified by Patzak (2001) for hop. In the second and third year, DNA was isolated from young leaves by DNeasy Plant Mini kit and enclosed protocol (Qiagen, Hilden, FRG).

PCR amplification and gel electrophoresis

Specific male-associated PCR marker by Polley et al. (1997) was used for sex identification in F_1 progenies.

PCR reaction mix (25 µl) was composed from 1× reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.75 U thermostable Taq DNA polymerase (Qiagen, Hilden, FRG), 30 ng of each primer (STSF: 5' ACAGAGTACAACTCAGAAACAAACC 3', STSR: 5' AAGGTCGCACAATGACCG 3') and 50 ng of genomic DNA. Thermocycling program consisted of an initial 3 min denaturation at 94°C, 35 cycles of 30 s at 94°C, 60 s at 54°C and 90 s at 72°C, ending with final elongation at 72°C for 10 min. PCR products were separated on 1.5% agarose gels (LMP, Bio-Whittaker Molecular Application, Rockland, Maine, USA) in TBE buffer at 2 V/cm voltage, stained by EtBr and visualized on UV transilluminator.

Sex identification and statistic evaluation

Number of female and male individuals was determinated within each F_1 progeny during flowering in field conditions. Correspondence analysis of sex segregation and molecular marker identification was statistically evaluated by χ^2 test (Havránek 1993). Statistic evaluation was analysed by Microsoft® Excel 2000.

RESULTS

For the initial experiments, selected female and male plants of four F_1 progenies (Sm99 H30, Sm99 H35, Sm99 H39, Sm99 H45) were used. Sex of these plants was identified before PCR analysis. Specific male-associated PCR marker, which length was 1.15 kb (Figure 1), was amplified in all male plants of crossing Sm99 H30 and Sm99 H39 (Table 1). PCR marker was not amplified in all female plants of four crossing used for initial experiments. Unfortunately, this marker was not amplified in one male plant of crossing Sm99 H35 and two male plants of crossing Sm99 H45 (Table 1). Statistic analysis confirmed that sex ratios of these F_1 progenies were significantly coincident with probability 0.71 and 0.5, respectively (Table 1). These results indicated that it was possible to use this specific PCR marker in analysis of small seedlings of breeding materials.

In total, 770 genotypes of F_1 progenies of three crossings (Sm00 H20, Sm01 H28 Sm01 H29) were used for experiments during two years. Very young seedlings of all F_1 progenies were used for PCR analysis. All tested plants were planted into breeding hopgarden. Sex identification was carried out during flowering. When some plants were without flowers in the first year of vegetation, they were described next year. 170 female and 40 male plants were identified in F_1 progeny of crossing Sm00 H20, which was 81% and 19%, respectively (Table 2). 107 female and 63 male plants were identified in F_1 progeny of crossing Sm01 H28, which was 63% and 37%, respectively (Table 2). 202 female and 138 male plants were identified in F_1 progeny of crossing Sm01 H29, which was 59.4% and 40.6%, respectively (Table 2). Very similar results were obtained by PCR analysis. 165 female and 45 male plants were identified in F_1 progeny of crossing Sm00 H20, which was 78.6% and 21.4%, respectively (Table 2). 111 female and 59 male plants were identified in F_1 progeny of crossing Sm01 H28, which was 65.3% and 34.7%, respectively (Table 2). 210 female and 130 male plants were identified in F_1 progeny of crossing Sm01 H29, which was 61.8% and 38.2%, respectively (Table 2). PCR marker was not amplified in some male plants of F_1 progenies of crossing Sm01 H28 (4 males) and Sm01 H29 (8 males). In contrast, PCR marker was amplified in 5 female plants of F_1 progenies of crossing Sm00 H20. Statistic analysis of sex ratios confirmed that PCR marker analysis and inspection of plants during flowering were significantly coincident with probability 0.4 for F_1 progeny of crossing Sm00 H20, 0.52 for F_1 progeny of crossing Sm01 H28 and 0.37 for F_1 progeny of crossing Sm01 H29 (Table 2). These results indicated that the use

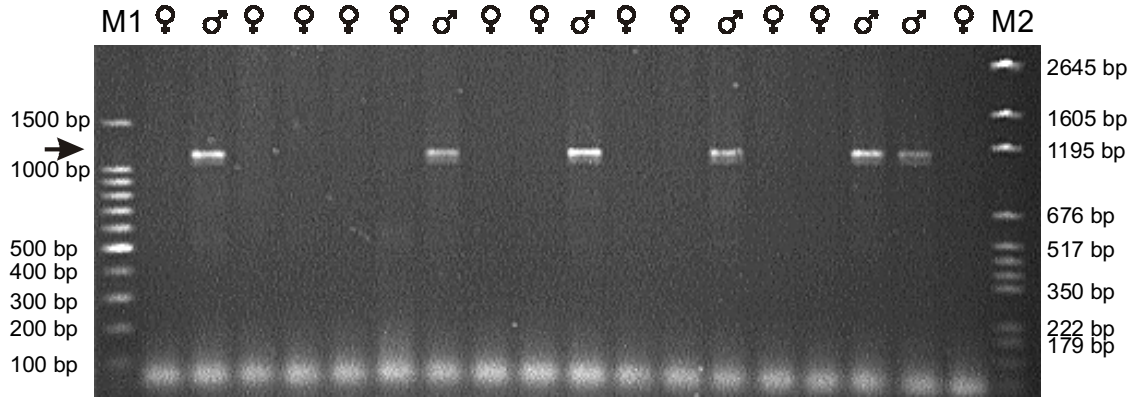

Figure 1. Analysis of PCR products in 1.5% agarose gel; specific PCR male marker is labelled by arrow; M1 – 100 bp Ladder, M2 – pGEM DNA marker (Promega, Madison, Wisconsin, USA)

Table 1. The evaulation of sex in F_1 progenies of four crossings by specific PCR molecular marker and inspection of plants during flowering in field conditions

Crossing	Sex identification by	Females	Males	χ^2 value	Probability
Sm99 H30	inspection of plants	14	2	0.0	$P = 1$
	PCR marker	14	2		
Sm99 H35	inspection of plants	27	10	0.137	$P = 0.71$
	PCR marker	28	9		
Sm99 H39	inspection of plants	12	6	0.0	$P = 1$
	PCR marker	12	6		
Sm99 H45	inspection of plants	18	17	0.457	$P = 0.5$
	PCR marker	20	15		

of specific PCR marker for F_1 progeny analysis successfully identified sex of juvenile plants and effectively improved breeding process.

DISCUSSION

In the initial experiments, we used specific male-associated PCR marker by Polley et al. (1997) for sex identification in selected plants of four F_1 progenies. We intended to find out, whether this specific PCR marker could be used for sex identification in different hop material of breeding processes in the Czech Republic. Čerenak and Javornik (1999) analysed 10 male plants by means of this molecular marker, which was amplified in all male plants with exception of one Japanese male plant. Wild hop from Japan (*Humulus lupulus* var. *cordifolius*) and derived materials have a multiple sex-chromosome system (Parker and Clark 1991, Dellaporta and Calderon-Urrea 1993). This sex system arises translocations between sex chromosomes and autosomes. It is possible that as follows, these changes some locus of chromosomes could be lost or locus with sequence of specific PCR marker could be absent on Y chromosome. It could be same case, why this marker was not amplified in one male plant of crossing Sm99 H35 and two male plants of crossing Sm99 H45. Parker and Clark (1991) reported that spontaneous interchanges also have occurred in *Humulus lupulus* during metaphase-I of meiosis, which obviously caused these sequence differences in segregated F_1 progenies. It was not conceivable that Y chromosome was absent in male plants, because the existence of XX-XO sex determination system has not been demonstrated in plants (Dellaporta and Calderon-Urrea 1993).

In the next experiments, we first attempted to analyse juvenile hop plants of F_1 progenies. In total, we analysed 770 genotypes of F_1 progenies of three crossings. There were from 19.0% to 40.6% of male plants in F_1 progenies. Nesvadba et al. (1999) reported that number of male hop plants ranged from 29% to 35.2% in F_1 progenies. Neve (1991) also reported that the ratio of female: male plants was about 2:1 and he suggested that excess of female plants was caused by pollen competition. Nesvadba et al. (1999) suggested that this ratio was caused by segregation of four sex chromosomes, which was determinated by quadrivalents of sex chromosomes and autosomes in American hops (Neve 1991). The amplified differences were found in 4 male plants of crossing Sm01 H28, 8 male plants of crossing Sm01 H29 and 5 female plants of crossing Sm00 H20. The sequence differences of Y chromosome in male plants could be due to the same mechanisms, which were described above. The amplification of specific PCR marker in female plants could be due to presence of this sequence on X chromosome, because Polley et al. (1997) reported that this sequence belonged to repeated DNA sequences and weakly hybridised to female plants. The next explanation could be presence of Y chromosome and two X chromosomes, which actively regulated sex determination of hop (Dellaporta and Calderon-Urrea 1993). The epigenetic control of sex determination, which was be due to methylation pattern of DNA (Janoušek et al. 1996), also could caused amplification of male-specific PCR marker in female plants. In spite of these differences, statistic analysis

Table 2. The evaluation of sex in F_1 progenies of three crossings by specific PCR molecular marker and inspection of plants during flowering in field conditions

Crossing	Sex identification by	Females (%)	Males (%)	χ^2 value	Probability
Sm00 H20	inspection of plants	170 (81.0)	40 (19.0)	0.707	$P = 0.4$
	PCR marker	165 (78.6)	45 (21.4)		
Sm01 H28	inspection of plants	107 (63.0)	63 (37.0)	0.415	$P = 0.52$
	PCR marker	111 (65.3)	59 (34.7)		
Sm01 H29	inspection of plants	202 (59.4)	138 (40.6)	0.797	$P = 0.37$
	PCR marker	210 (61.8)	130 (38.2)		

confirmed that sex ratios of all F_1 progenies were significantly coincident.

We demonstrated that specific PCR marker identified sex of juvenile hop plants in F_1 progenies. Some minor differences, which were found, could be resolved by new marker system developed on male-specific AFLP markers of hop male linkage map in the future (Seefelder et al. 2000). Nevertheless, the utilization of this molecular marker significantly reduces the time for evaluation of plants, permits the separation of both sexes and considerably reduces the amount of labour and field space.

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ABSTRAKT

Identifikace pohlaví v potomstvech F_1 generací chmele (*Humulus lupulus* L.) pomocí molekulárního markeru

Identifikace pohlaví chmele (*Humulus lupulus* L.) jako dvoudomé rostliny je velice důležitá pro šlechtitelský proces. Použití molekulárních metod pro selekci podle genetických markerů (MAS) poskytuje rychlou a spolehlivou identifikaci pohlaví v populacích F_1 generací. Nejprve jsme prokázali použití specifického PCR molekulárního markeru pro hodnocení pohlaví u vybraných rostlin. Tento marker nebyl amplifikován pouze u 3 z 35 samčích rostlin. Dále jsme úspěšně analyzovali 770 genotypů populací F_1 generací tří křížení. Amplifikované rozdíly byly nalezeny u 4 samčích rostlin křížení Sm01 H28, 8 samčích rostlin křížení Sm01 H29 a 5 samičích rostlin křížení Sm00 H20. Statistická analýza potvrdila, že poměr pohlaví v populacích všech F_1 generací byl prokazatelně shodný. Získané výsledky potvrdily, že tento specifický PCR marker úspěšně identifikuje pohlaví juvenilních rostlin chmele v populacích F_1 generací a efektivně vylepšuje šlechtitelský proces.

Klíčová slova: PCR; selekce podle genetických markerů (MAS)

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