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Random amplified polymorphic DNA (RAPD) markers in hop, *Humulus lupulus*: level of genetic variability and segregation in F_1 progeny

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Abstract The level of genetic variation in 24 hop genotypes was studied using the recently developed technique for producing random amplified polymorphic DNAs (RAPDs). Of the 60 primers screened, eight produced polymorphic RAPD bands, 38 produced bands that were monomorphic for all genotypes and 19 did not produce any amplification product. It appeared that the level of polymorphism among the genotypes was generally low. Three of the primers, A11, A17 and C9, were used to determine the stability and segregation of RAPD markers in five families with a total of 182 F_1 progeny. The segregation ratios of these markers in the F_1 progeny suggested that they were inherited in a Mendelian manner. RAPD markers were stable and may be useful for the construction of linkage maps in hop.

Key words Random amplified polymorphic DNA · Hop · Genetic variation

Introduction

Humulus lupulus (hop) is a dioecious, perennial, climbing plant that is grown in many countries for its value in the brewing industry. The value of hops lies in the lupulin glands which contain resins and essential oils that provide aroma and flavor to beer and ale (Neve 1991). Lupulin glands are found in abundance in the inflorescences of female plants, while male flowers contain very few of them. The effort of hop breeders has been directed primarily at increasing the levels of the essential brewing components of hop while introducing and incorporating superior agronomic characters such as resistance to diseases and pests. Hop breeding is complicated by the fact that male plants are of no commercial value. Consequently, hop breeders cannot select suitable male genotypes for a breeding program. In general, female plants are open pollinated and selection for a character(s) is (are) made from within a seedling family, usually the F_1 generation.

Despite the economic importance of hops, the extent of genetic variability within the species is virtually unknown. There are no well established cytogenetic, isozyme or morphological markers for the crop. Although there is substantial intraspecific variation in vegetative morphology, especially leaf characters, it is not possible to distinguish cultivars/genotypes on their external morphology alone. Further, these phenotypic characters are generally influenced by environmental factors and the growth stage of the plant and are therefore unreliable as criteria for identifying cultivars or genotypes.

The development and application of new technologies such as (RFLPs) restriction fragment length polymorphisms (Tanksley et al. 1989) and (RAPDs) random amplified polymorphic DNAs (Williams et al. 1990) have provided new DNA markers that are useful in genetic mapping, for improved selection in breeding programs and for the cloning of genes (Kazan et al. 1993). Our attempts at developing RFLP maps in a set of 24 diverse hop genotypes, some of which were under selection for specific traits, was hampered by the very low level of DNA polymorphism (Pillay and Kenny, unpublished data). However, in preliminary experiments the same set of genotypes did show some differences in RAPD banding patterns which may be potentially useful in the construction of linkage maps. The RAPD technique has become a powerful approach for detecting genetic variation among individuals within a plant species. The technology relies on the amplification of unspecified regions of genomic DNA by the polymerase

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chain reaction (PCR) using short primers of arbitrary nucleotide sequence (Williams et al. 1990). The RAPD technique is considered to have many advantages over RFLPs and provides a fast, efficient method for scoring large numbers of genetic markers. It has a wide range of applications including genetic mapping (Rafalski et al. 1991; Reiter et al. 1992), phylogenetic analysis (Jarret et al. 1993; Stiles et al. 1991; Wilkie et al. 1993; Link et al. 1995), the construction of linkage maps (Michelmore et al. 1991; Ohmori et al. 1995), evaluating gene flow between species (Arnold et al. 1991), identifying individuals, cultivars or species by genomic fingerprinting (Keil and Griffin 1994; Mailer et al. 1994; Abo-elwafa et al. 1995; Lanham et al. 1995; Perron et al. 1995; Schnell et al. 1995), and the identification of parents in pedigree analysis (Welsh et al. 1991; Tinker et al. 1993). Recently, a RAPD marker was linked to sex determination in *Pistacia* (Hormaza et al. 1994), a feature of potential interest to hop breeders. Determining the gender of hop plants at an early growth stage would be advantageous to growers since males are not cultivated.

Before making extensive use of RAPD markers in a breeding program, the stability and genetics of these markers from parents to offspring need to be evaluated. In some studies it was shown that a proportion of RAPD markers did not obey the rules of simple Mendelian inheritance. For example, in *Arabidopsis* only 57% of 392 RAPD markers segregated in a Mendelian fashion (Reiter et al. 1992); in diploid alfalfa 76% of 37 polymorphic markers segregated as dominant Mendelian markers (Echt et al. 1992). Carlson et al. (1991), found that only 70% of RAPD markers in F_1 progenies of conifers were inherited as dominant markers. In corn about 10% of the RAPD markers were inherited in a non-Mendelian manner (Heun and Helentjaris 1993). In *Stylosanthes*, about 25% of the RAPD markers either did not segregate in the F_2 population or deviated significantly from an expected 3:1 segregation ratio (Kazan et al. 1993). It is expected that RAPD bands of maternal DNA origin would show non-Mendelian inheritance. However, there is no suitable explanation for non-Mendelian inheritance of RAPDs arising from the nuclear genome. RAPD markers that do not follow basic genetic models may not be useful in breeding.

The objectives of the present study were to (1) examine the utility of RAPD markers in determining molecular genetic variation in hop and (2) report on the segregation patterns and inheritance of RAPD bands in F_1 progeny of hop to determine the suitability of these markers for a breeding program.

Materials and methods

Plant material

The hop (*Humulus lupulus* L.) plants used in this study are listed in Table 1. These plants are maintained at the Irrigated Agriculture Research and Extension Center, Prosser, Washington, USA. The cultivars represent selections that were made for brewing quality and other agronomically useful characteristics including resistance to hop aphid, high alpha-acid and essential oil content, and high yield potentials. Prior to this investigation, crosses were made between specific cultivars in a program breeding for aphid resistance and brewing quality traits. We chose some of these crosses for segregation analysis of RAPD markers after determining present/absent band differences between the parents. These polymorphic bands were used to monitor the segregation of RAPD markers in five crosses with a total of 180 F_1 progeny. For each cross and primer combination, we scored the presence or absence of the RAPD marker in the F_1

Table 1 List of hop genotypes/cultivars used to examine variation in RAPDs, together with their gender, description and origin. M and F represent male and female genotypes

Genotype	Gender (M or F)	Description cultivar/wild	Origin
21015	F	Tettnanger	European
21051	F	Apollon	European/North American [a]
21116	F	Brewers Gold	European/North American [a]
58016	F	Wild	Native American
60033	F	Wild	Native American
62013	F	Comet	European/North American [a]
65102	F	L1Cluster	European/North American [a]
66055	F	First Choice	North American [b]
8254-181	F	*	European [c]
8254-181	F	*	European [c]
21087	M	Wild	Native European
21090	M	Wild	Native European
21110	M	*	European/North American
21132	M	*	European/North American
21184	M	*	North American [b]
21234	M	*	European [d]
21235	M	*	European [d]
21237	M	*	European [d]
60028	M	*	North American
8658-039	M	*	European/North American [a]
8659-023	M	*	European
8659-045	M	*	European
8685-014	M	*	European/North American [a]
8693-043	M	*	European/North American [a]

[a] Genotypes of mixed origin (European and North American)
[b] Genotypes of North American and unknown origin
[c] Genotypes of European plus unknown origin
[d] Genotypes of possible European origin
* Breeding stocks maintained at the Irrigated Agricultural Research and Extension Center, Prosser, Washington, USA

progeny. A summary of the details, including the primers used, the parents and assumed genotypes and the observed segregation and probabilities, are summarized in Table 2.

DNA isolation and polymerase chain reaction

Total DNA was isolated from young leaves of the parents and progeny plants by the CTAB method (Saghai-Maroof et al. 1984; Doyle and Doyle 1990). The concentration of the DNA samples was determined by ultraviolet absorbance at 260 nm in a Varian DMS 200 spectrophotometer and the DNA samples were adjusted to 0.2–0.5 µg/µl by diluting with TE (10mM Tris, 1mM EDTA) buffer. Approximately 25 ng of DNA was used in 100 µl-volume amplification reactions containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.05 mM each of dATP, dCTP, dGTP and dTTP (Stratagene, La Jolla, Calif., USA), 1 µM random primer (Operon Inc., Alameda, Calif) and 2–5 units of *Taq* DNA polymerase, initially purchased from Stratagene (La Jolla, Calif.) and subsequently obtained from Promega (Madison, Wis., USA). The reaction mixture was overlaid with 60 µl of mineral oil. The PCR reaction was done in a thermal cycler (Coy TempCycler II and Perkin Elmer Cetus 480) with the following amplification conditions: an initial 4-min denaturation at 94 °C followed by 45 cycles of 1 min at 94 °C, 1 min at 42 °C and 2 min at 72 °C with a final extension step at 72 °C for 8 min. Approximately 20 µl of the amplification products was analyzed by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and photographed on an ultraviolet transilluminator. A 1-kb ladder marker (Gibco-BRL, Bethesda, M. USA) was used as a molecular-weight standard.

Hybridization analysis

Amplified DNA fragments were transferred to nylon membranes using the high-alkaline medium (0.4 M NaOH, 1 M NaCl) described by Reed and Mann (1985). The fragments to be used as probes were excised from duplicate gels and the DNA was extracted using the Geneclean II kit (Bio 101, Inc. La Jolla, Calif). These fragments were radiolabelled with ^{32}P-dCTP by random priming (Feinberg and Vogelstein 1983). Hybridization and autoradiographic procedures were carried out as described in Pillay (1993).

Results

RAPD variation

To determine the suitability of the RAPD technique to identify DNA polymorphisms among the 24 hop genotypes, 60 10-mer primers from kits A, B and C (Operon Technologies) were used in PCR reactions. From the 60 primers screened, eight produced polymorphic banding patterns among some of the genotypes. The size of the amplification products ranged from approximately 500 bp to 2036 bp. An example of the amplification products obtained with primers A11 and C8 is shown in Fig. 1. Banding patterns were characterized as being very intense, light, and faint. No RAPD band was found to be specific to any genotype, although genotypes sharing a common ancestry were found to share unique banding patterns. Thirty three of the primers produced RAPD bands that were monomorphic across all the genotypes while 19 did not produce any amplified products. The level of polymorphism among the genotypes was generally low in comparison with other studies (see Discussion). The observed variation did not change appreciably even under altered PCR conditions. For example, lowering the annealing temperature produced very diffuse bands of decreased intensity.

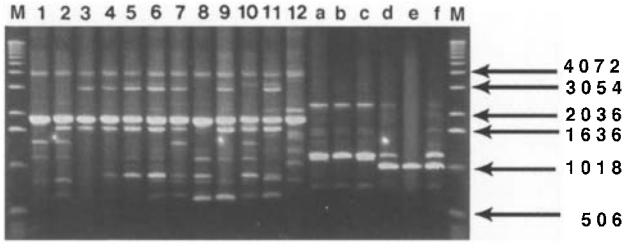
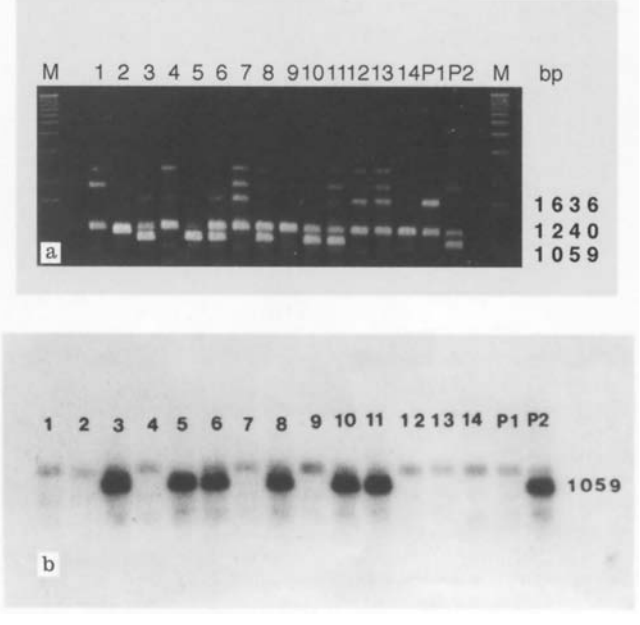


Fig. 1 RAPD patterns obtained from hop genotypes using primer C8 (*lanes 1–12*) and primer A11 (*lanes a–f*). The sizes of the molecular-weight standards (*M*) are shown in bp

Segregation analysis

Three primers, A11, A17 and C9, were used in determining the patterns of inheritance of RAPD markers. The RAPD patterns obtained with primer A11 for the cross 21116 × 8658-039M and for some of the F_1 progeny are shown in Fig. 2 a. The 1059-bp fragment was considered for analysis. This fragment was absent in parent P1 but present in P2 and in 6 of the 14 F_1 progeny (Fig. 2 a). Figure 2 b shows the autoradiograph of the Southern blot from Fig. 2 a after probing with the 1059-bp fragment. Since the 1059-bp fragment was absent in P1, it was assigned the genotype *aa* (absence of the fragment) and the *Aa* genotype (presence of a fragment) was

Fig. 2a,b Gel electrophoresis and autoradiograph of Southern blot of RAPD fragments in the cross 21116 × 8658-039. **a** RAPD fragments in progeny (*lanes 1–14*) and parents (*P1 and P2*) with primer A11. **b** Autoradiograph of Southern blot after hybridization of the 1059-bp fragment of parent 21116 (*P2*) to the RAPDs shown in **a**



deduced for parent P2. The observed frequencies of presence versus absence of the fragment corresponded to the expected 1:1 F_1 ratio from crossing *aa* × *Aa* (Table 2). Figure 3 a and b shows the RAPDs and Southern hybridization results obtained when the presence and absence of the 1636-bp fragment in the cross 58016 × 8658-039M was considered for analysis. In Figure 4, four of the RAPD markers in the cross 21015 × 86840-014 considered for segregation analysis are shown. The F_1 segregation analysis of all the RAPD markers was treated in a similar manner and putative genotypes, observed segregation numbers, and probabilities are summarized in Table 2.

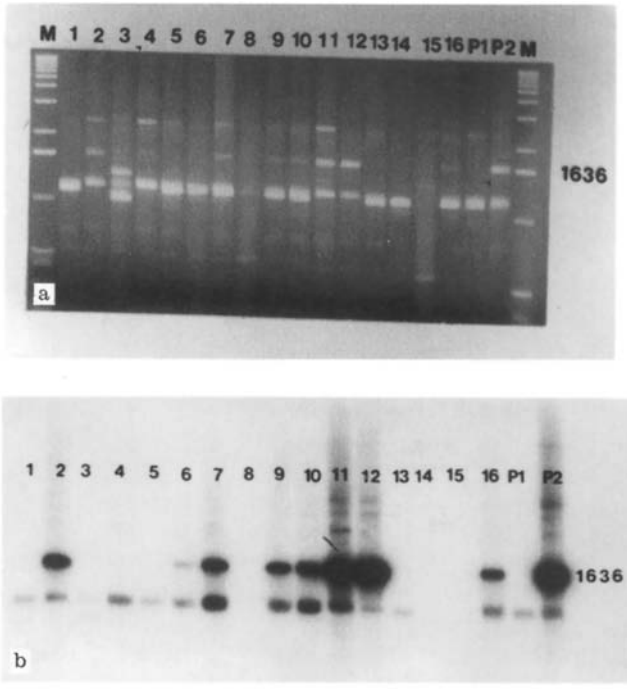


Fig. 3a,b Gel electrophoresis and autoradiograph of Southern blot of RAPD fragments in the cross 58016 × 8658-039. **a** RAPD fragments in progeny (*lanes 1–16*) and parents (*P1 and P2*) with primer A11. **b** Autoradiograph of Southern blot of the 1636-bp fragment of parent 8658-039 (*P2*) to the RAPDs shown in **a**

Discussion

RAPD variation

The RAPD patterns (Fig. 1) revealed low levels of genetic variation among the 24 diverse hop genotypes that included cultivated and wild hop of European and North American origin. Knowledge of the amount of genetic variability in a crop species is important for any breeding program because it provides the basis for the development of new genotypes. The low level of DNA polymorphism among the hop genotypes, coupled with the large number of common bands, implies that the genomes of the sample under study are rather homogeneous.

Similar results have been noted in other studies at the intraspecific level, especially in *Arachis hypogaea* (Halward et al. 1992) and *Allium cepa* (Wilkie et al. 1993). By contrast, a high level of polymorphism was observed at the same level in *Musa acuminata* (Jarret et al. 1993) and chrysanthemum (Wolff and Peters-Van Rijn 1993). The reason for this differential rate of variation in RAPD patterns at the intraspecific level is unknown. There is no available information on the origin of hop cultivation and the source of the original plants (Neve 1991). However, the homogeneity of the genome in cultivated hop is probably explainable by early hop breeding practices. It is believed that many new hop varieties were selections that were made from within local populations (Neve 1991). These phenotypically superior plants were then propagated and released as new or improved varieties often with new names reflecting either the growers

Table 2 Segregation analysis of RAPD markers in hop showing primers, fragments analyzed, parents and genotypes, observed genotypes in F_1 and probabilities

Primer	Fragment size (bp)	Parents and assumed genotypes	Observed F_1		Probability
			Dominant	Recessive	
		21116 × 8658-039	AA + Aa	aa	
A11	1636	*aa* × *Aa*	23	30	0.32
	1059	*bb* × *Bb*	24	29	0.48
		8254-146 × 8658-039			
A11	1636	*aa* × *Aa*	12	16	0.48
A17	2036	*CC* × *cc*	28	0	–
		58016 × 8658-039			
A11	1636	*aa* × *Aa*	15	14	0.90
		21116 × 60028			
A11	1228	*Dd* × *dd*	15	13	0.68
		21015 × 8685-014			
C9	1759	*ee* × *Ee*	24	20	0.58
	1678	*Ff* × *ff*	20	24	0.58
	1372	*Gg* × *gg*	21	23	0.78
	574	*hh* × *Hh*	25	19	0.80

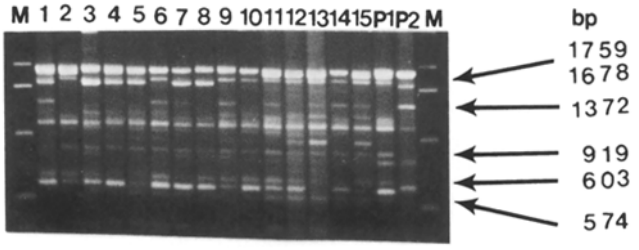


Fig. 4 RAPD fragment patterns from two parents *P1* (8685-014) and *P2* (21015) and from 15 individuals of the F_1 progeny using primer C9. The *arrows* point to segregating RAPD markers

or the district of origin. Moreover, since hop is clonally propagated it was introduced to different countries where a cultivar sometimes assumed a different name. For example, Styrian Golding, the primary cultivar in Slovenia, is presumed to be a Fuggle introduced from England (Neve 1976). Interestingly, RAPD patterns did not differ in the wild and cultivated forms suggesting that the hop genome has not changed appreciably during domestication. The absence, or low level, of genetic variation in hop may also be explained by founder events or bottleneck effects.

Despite the low levels of variation observed in RAPD patterns, it was interesting to observe that genotypes sharing a common ancestry also shared unique bands. For example, a unique fragment of approximately 1350 bp was found in Brewer's Gold (21116), Appolon (21051) and wild (58016, 60033) North American hop with primer C6. It is known that Brewer's Gold was selected from seedlings from an open-pollinated cross of a wild hop from Manitoba, Canada (Neve 1991). Brewer's Gold is directly involved in the ancestry of Apollon. The unique band present in these genotypes implies that, with further analysis, RAPDs may be useful for addressing phylogenetic questions in hop.

The RAPD reaction is affected by a number of experimental conditions (Carlson et al. 1991; Hosaka and Hannemann 1994). The stability of RAPD markers and the repeatability of the PCR reaction has been a source of concern in many studies (Devos and Gale 1992; Wilkie et al. 1993; Mailer et al. 1994). Similar concerns were considered in the present study. Therefore, to ensure that RAPD banding patterns were consistent and reproducible for an individual, each PCR reaction was repeated at least three times. Further, only those major bands whose presence or absence could be easily distinguished were used to follow the segregation patterns in the progenies. In some cases, PCR bands in some progeny lanes appeared faint. In order to confirm the homology of the DNA bands having identical molecular weights, PCR reactions were followed by Southern hybridization. This additional experimental step is necessary to remove the ambiguity of scoring faint bands as being present/absent and verifies the homology of similar-sized DNA bands from the same primer. Verification of the homology of RAPD bands is largely ignored by many researchers. In hop, Southern hybridization of PCR gels has shown that bands with identical molecular weight are not necessarily homologous (Pillay and Kenny 1995).

Segregation of RAPDs

Previous studies analyzing the segregation of RAPDs (Echt et al. 1992; Roy et al. 1992) have put forth two assumptions concerning these markers: (1) each band is considered to represent a single locus and is (2) a dominant marker for that locus. On this basis, the presence of a fragment in a parent is considered to be either in the homozygous dominant or the heterozygous condition. It is not possible to distinguish homozygosity from heterozygosity at a locus from the presence of a RAPD band (Carlson et al. 1991). A parent not containing a fragment is assigned the recessive genotype. When a marker is present in one parent and absent in the other ($Aa \times aa$ or $aa \times Aa$) the marker will segregate in a 1:1 ratio in the F_1 generation. When a marker is present in both parents ($Aa \times Aa$) the expected segregation ratio for that marker in the progeny is 3:1. We have analyzed our data within the framework of these assumptions. All the RAPD markers in the F_1 progenies of hop (Table 2) segregated in ratios that were consistent with Mendelian inheritance and all the markers were dominant. Chi-square tests were performed for each of the markers to determine if segregation differed from the expected 1:1 ratios. At a significance level of $P = 0.05$ all the tests, except one, were in agreement with the expected ratios. In the one exception, involving a 2036-bp fragment in the cross 8254-146 × 8658-039, the dominant parent was assumed to be homozygous. In comparison with other studies, RAPD markers in hop did not deviate from normal biparental inheritance. Similar results have been observed in other species including Douglas fir and white spruce (Carlson et al. 1991), broccoli and cauliflower (Hu and Quiros 1991), lettuce (Michelmore et al. 1991), tomato (Giovannoni et al. 1991), canola (Deragon and Landry 1992) and *Betula* (Roy et al. 1992).

In some progeny, new bands not found in either parent were observed. These bands could have arisen in several ways: (1) they may be the result of recombination that produced new binding sites for the primers in the DNA of the F_1 progeny (2) amplification is possible even when the primer is not perfectly matched with the template DNA (Williams et al. 1990; Rafalski et al. 1991), and (3) competition for primer-binding sites is an important factor controlling which RAPD fragments are sufficiently amplified to be be visible (Hunt and Page 1992).

In the absence of other genetic markers, the present study showed that RAPDs are potentially valuable markers for a hop breeding program. A method that produces consistent and stable RAPD patterns in hop has been established. We have not as yet been able to link any of the RAPD markers with a trait of interest. This study has, however, provided the basis for such research. Polymorphic RAPD markers in parental

plants segregated as independent alleles in the F_1 progeny of hop suggesting that RAPDs have a genetic basis and may be useful in linkage mapping.

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