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L. Hartl · S. Seefelder

Diversity of selected hop cultivars detected by fluorescent AFLPs

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Abstract The amplified fragment length polymorphism (AFLP) technique was used to assay eight hop cultivars. The application of fluorescent-labelled primers proved to be a valuable tool and substituted radiolabelling. Digestion with the enzymes *Eco*RI/*Mse*I and amplification with primers having three selective bases at the 3'end resulted in distinct banding patterns for imaging with a fluorescent scanner. A total of 523 AFLP fragments derived from eight primer combinations were analysed. On average, 18 polymorphisms per combination were displayed. The Saazer "noble" hop cultivars 'Saazer', 'Tettnanger' and 'Spalter' could not be discriminated. The lowest genetic similarity (GS) between lines was computed for the bitter hops 'Hallertauer Magnum' and 'Wye Target': GS value of 0.89. The high level of genetic similarity of the analysed hop cultivars is discussed.

Key words AFLP · Fluorescence · Hop · *Humulus lupulus* · Genetic distance

Introduction

Hops (*Humulus lupulus*), a member of the *Cannabaceae* family, is an essential crop for the brewing industry. The female flowers are used as a bittering and preservative agent as well as to give flavour to beer. Hops is dioecious and mainly vegetatively propagated. Early breeders concentrated only on clonal selection from land varieties to develop varieties like 'Hallertauer Mittelfrüher' and 'Saazer'. Nowadays, the classical hop breeding programme is based on specific sexual combinations and subsequent, selection of superior individuals among the segregating progeny. Therefore, it is essential to secure and enlarge the genetic diversity of the breeding material to generate new, improved cultivars.

Up until now, morphological parameters and quality data have been studied to determine genetic diversity and to identify cultivars. A major advantage of employing DNA markers is that they are not influenced by environmental effects or epistatic interactions. They detect variation at the level of the DNA sequence and have proved to be an extremely effective tool for distinguishing between closely related genotypes. In several species different marker types have been used to estimate the genetic diversity of cultivars and wild ancestors. The recently developed AFLPs (amplified fragment length polymorphisms) (Zabau and Vos 1993) have great potential as a new marker technique because (1) a high level of polymorphisms can be detected, (2) no prior sequence information is necessary and (3) many markers can be analysed in a short time. AFLPs are generated by amplifying restriction fragments using primers complementary to ligated adapters. Selective nucleotides at the 3'end of the primers reduce the number of amplified restriction fragments and make them resolvable in a standard sequencing gel. So far AFLPs have been used to estimate genetic relationships in lentil (Sharma et al. 1996), soybean (Maughan et al. 1996), lettuce (Hill et al. 1996) and common bean (Tohme et al. 1996).

Hitherto, hops was rarely investigated with respect to its cytogenetic and genomic constitution. Randomly amplified polymorphic DNA (RAPD) analysis was carried out on hop lines of various origins to differentiate the lines at the molecular level (Pillay and Kenny 1996). In comparison to RAPDs amplified microsatellites provided a higher level of polymorphism for the discrimination of breeding lines (Brady et al. 1996). But the disadvantages of microsatellite markers are the

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L. Hartl (✉) · S. Seefelder
Bayerische Landesanstalt für Bodenkultur und Pflanzenbau (LBP), Biotechnologie, Vöttingerstr. 38, 85354 Freising, Germany
Fax: +49 (08161) 71-4514
E-mail: lorenz.hartl@lbp.bayern.de

requirement of genomic libraries, sequencing of clones and the synthesis of primers. Specific sequences for the Y chromosome have been identified with RAPDs, and a derived sequence-tagged-site (STS) marker was used to determine the sex of the hop plants (Polley et al. 1997).

The objective of the study presented here was to apply AFLP analysis to a small subset of important hop cultivars and to investigate the efficiency of this marker type in evaluating the genetic diversity. As an alternative to radioactive labelling, fluorescence-labelled primers in combination with a fluorescence scanner were used to visualize the AFLP pattern.

Materials and methods

Plant material

Eight hop cultivars from the breeding garden of Hüll (Germany) were used. The aroma varieties 'Hallertauer Mittelfrüher' and 'Perle', the "noble" hops 'Saazer', 'Spalter', 'Tettnanger' and the bitter hops 'Northern Brewer', 'Hallertauer Magnum' and 'Wye Target' were chosen to represent lines with a closer and a more distant ancestry. They are part of actual breeding material.

DNA isolation

Young leaves were collected, freeze-dried and ground to powder using a mechanical mill. Total DNA was isolated based on the procedure described by Doyle and Doyle (1990). DNA concentration was estimated in comparison with known concentrations of lambda DNA in a 1% agarose gel.

AFLP analysis

AFLPs were performed as described by Keygene (Zabau and Vos 1993; Vos et al. 1995) with minor modifications. Adapters and primer sequences are shown in Table 1. Genomic DNA (0.5 µg) was first digested with 5 units of both *Eco*RI (Pharmacia) and *Mse*I (New England Biolabs) for 1 h and then 500 pmol *Mse*I adapter and 50 pmol *Eco*RI adapter were ligated to the restriction fragments with 1 unit T4-ligase (Pharmacia) for 3 h. Restriction and ligation were performed in 1 × RL buffer at 37°C (10 m*M* TRIS.HAc pH 7, 5, 10 m*M* MgAc, 50 m*M* KAc, 0.5 m*M* DTT, 50 ng/µl BSA).

The complexity of the DNA was reduced by amplifying 1/40 of the ligation reaction in the preselective amplification step using primers with one selective base at each side in a total volume of 20 µl [6 pmol of E01 and M02 primer, 0.2 m*M* dNTPs, 1.5 m*M* $MgCl_2$, 0.5 units *Taq* (Boehringer), 1 × mol PCR (polymerase chain reaction) buffer]. The profile began with an incubation step of 2 min at 72°C, followed by 20 cycles of 30 s at 94°C, 1 min at 60°C and 2 min at 72°C. Selective amplification was conducted with three selective bases at the 3′end of both primers. The *Eco*RI primer was labelled with fluorescein at the 5′end. The assay was the same as that for the preselective amplification except for the template, which was equivalent to 1/130 of the preamplification reaction. After the first denaturation step (2 min, 94°C) a touch-down profile (65°C–1°C/cycle for 9 cycles) for the annealing step was added and continued with an annealing temperature of 56°C for the next 23 cycles. The following eight primer combinations were used for the estimation of genetic similarity: E37/M61, E38/M55, E38/M59, E38/M47, E33/M55, E33/M60, E33/M61 and E41/M59. All amplifications were performed in a 9600 Perkin-Elmer thermocycler.

Table 1 Adapter and primer sequences used for AFLP analysis

Primers/adapters		Sequences (5′ → 3′)
*Eco*RI-adapter		CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA
*Eco*RI + 1 primer	E01	GACTGCGTACCAATTCA
*Eco*RI + 3 primer	E33	E01 + AG
	E37	E01 + CG
	E38	E01 + CT
	E41	E01 + GG
*Mse*I-adapter		GACGATGAGTCCTGAG TACTCAGGACTCAT
*Mse*I + 1 primer	M02	GATGAGTCCTGAGTAAC
*Mse*I + 2 primer	M18	M02 + T
*Mse*I + 3 primer	M47	M02 + AA
	M55	M02 + GA
	M59	M02 + TA
	M60	M02 + TC
	M61	M02 + TG
*Mse*I + 4 primer	M224	M02 + TGT

Electrophoresis and gel analysis

The resulting reaction samples were mixed with an equal volume of loading dye (98% formamide, 10 m*M* EDTA, 0.025% bromphenol blue and 0.025% xylene cyanol). The samples were denatured for 3 min at 94°C, chilled on ice and run on a denaturating 5% polyacrylamide gel under standard sequencing conditions. After separation, the gel sandwich was scanned in the Fluorimager 595 (Molecular Dynamics). The scanning was performed under high-sensitivity conditions with an excitation wavelength of 488 nm and a band-pass filter (530 ± 15 nm). The resulting image was analysed with the software package FRAGMENT ANALYSIS 1.1 of Molecular Dynamics.

Data analysis

A binary data matrix reflecting the presence or absence of specific AFLPs was generated for the set of hop cultivars. It was assumed that each band of different size reflects a single locus. Only unambiguously scored bands were used in the matrix. The genetic similarities (GS) between line i and j were estimated using the formula of Dice (1945) as Gsij = 2Nij/(Ni + Nj), where Nij is the number of common loci between i and j, and Ni is the number of loci in i, respectively Nj is the number of loci in j. A dendrogram was generated using the unweighted pairgroup method average (UPGMA) clustering procedure. In addition, principle coordinate analysis (PCO) was performed to visualize the dispersion of the individuals in relation to the first two principle axes of variation. All computations were done using the procedures in NTSYS-pc (Rohlf 1991).

Results

AFLP primer combinations with different numbers of selective bases at the 3'end were compared to determine the number of selective bases which give the highest information content in a gel. *Eco*RI + 3 primers were

combined with *Mse*I + 2, *Mse*I + 3 and *Mse*I + 4 primers. *Mse*I + 2 primer combinations were ruled out because of the many bands that could not be separated clearly in a standard sequencing gel. A large number of defined bands was scored by the + 3 primer, whereas + 4 selective bases did not exploit the resolution capacity (Fig. 1A). Therefore, all further experiments were performed with *Eco*RI + 3 primers in conjunction with *Mse*I + 3 primers. The present study showed that the application of fluorescence-labelled primers resulted in a large number of distinguishable and scorable AFLP fragments.

Out of 60 primer combinations 8 were selected for their reliable banding pattern. An example of 2 combinations is shown in Fig. 1B. Only fragments which were obtained after repeating the experiment are shown. Between 37 and 87 bands were amplified with each of the 8 AFLP primer combinations. On average 0.5% of the clearly scorable fragments were absent or present in a repeated experiment. In total, 523 different-sized fragments were scored, 145 of them polymorphic. Within the eight cultivars 5–28 (18 on average) polymorphisms per primer combination were found. Nevertheless, no variation was observed between cvs 'Saazer', 'Spalter' and 'Tettnanger'.

Table 2 shows the estimation of genetic similarities between the genotypes based on the number of common fragments (Dice 1945). The GS values ranged from 1.0 within the "noble" hops from the Saazer aroma type to 0.89 for the pair 'Hallertauer Mittelfrüher' and 'Wye Target'. A dendrogram (Fig. 2A) was constructed using the similarity data matrix. To visualize the relationships in greater detail we calculated a principle coordinate analysis (Fig. 2B) which used two dimensions for the description of the data. Of the total genetic variation 73% could be explained by the first two

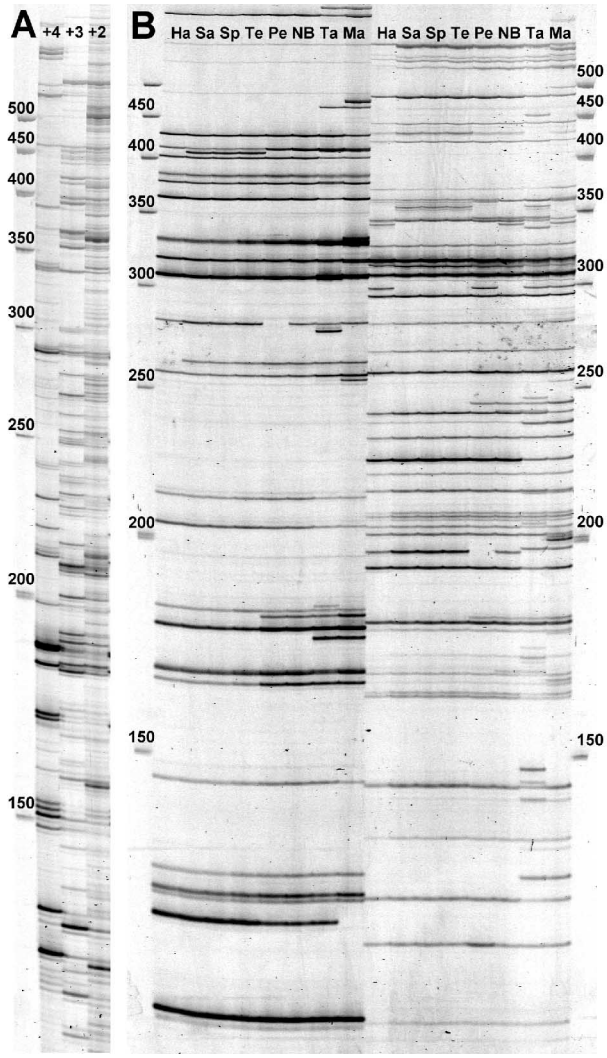

Fig. 1A, B AFLP analysis of hop. **A** The reactions of the hop cultivar 'Wye Target' (*Ta*) were generated with primer E33 combined with *Mse*I- primers with four (+ 4, M224), three (+ 3, M61) and two selective bases (+ 2, M18) at the 3′ end. **B** DNA fingerprinting of eight hop cultivars, 'Hallertauer Mittelfrüher' (*Ha*), 'Saazer' (*Sa*), 'Spalter' (*Sp*), 'Tettnanger' (*Te*), 'Perle' (*Pe*), 'Northern Brewer' (*NB*), 'Wye Target' (*Ta*) and 'Hallertauer Magnum' (*Ma*) was performed with primer combinations E38/M55 (*left side*) and E38/M59 (*right side*)

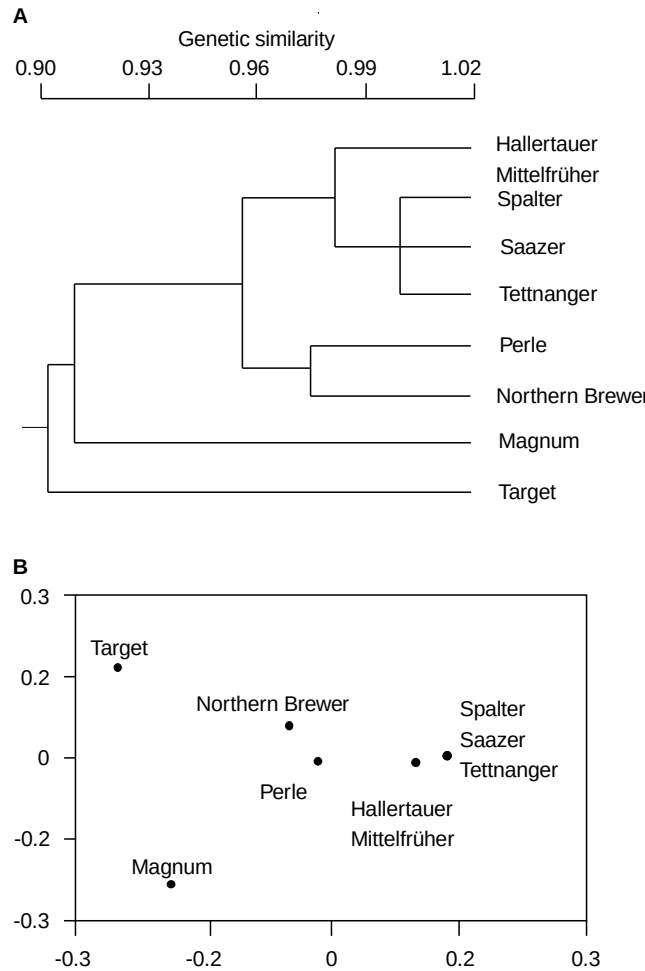

Fig. 2 Dendrogram (**A**) and principle coordinate analysis (**B**) of the eight hop cultivars derived from the genetic similarity matix

Table 2 Genetic similarity matrix for eight hop cultivars based on the formula of Dice (1945)

Genetic similarity	Hallertauer Mittelfrüher	Saazer	Spalter	Tettnagner	Perle	Northern Brewer	Wye Target	Hallertauer Magnum
Hallertauer Mittelfrüher	1.00							
Spalter	0.98	1.00						
Saazer	0.98	1.00	1.00					
Tettnanger	0.98	1.00	1.00	1.00				
Perle	0.96	0.96	0.96	0.96	1.00			
Northern Brewer	0.95	0.95	0.95	0.95	0.97	1.00		
Wye Target	0.89	0.89	0.89	0.89	0.91	0.93	1.00	
Hallertauer Magnum	0.91	0.90	0.90	0.90	0.93	0.91	0.90	1.00

eigenvalues. A main result of the analysis is the very close relationship between the Saazer "noble" hops and the wide genetic distance of cvs 'Hallertauer Magnum' and 'Wye Target' to all other lines.

Discussion

For generating AFLPs the use of fluorescence-labelled primers is an alternative to labelling with radioactivity or silver staining. In contrast to radiolabelling or silver staining fluorescence-labelled primers increase the flexibility of work flow because there is no decay period of radioactivity to be taken in account or the necessity of primer labelling or staining. The restriction enzyme combination *Eco*RI-*Mse*I and three additional selective bases of the primers are suitable for distinguishing hops. This has also been shown for other crop species such as barley (Becker et al. 1995), lettuce (Hill et al. 1996), and soybean (Maughan et al. 1996), common bean (Tohme et al. 1996) and sugar beet (Schondelmaier et al. 1996). Hops is believed to carry $2n = 20$ chromosomes (Neve 1991), but the DNA content of the genome has not been exactly determined. From the AFLP reaction pattern it is fair to assume that the organization of the hop genome is not extraordinarily different to previously analysed species.

Tohme et al. (1996) evaluated less than 3% different bands in their AFLP experiments with wild bean (*Phaseolus vulgaris*). Our error rates of the scored bands between two experiments were less than 1%, which is low, compared to RAPDs. From repetition experiments with RAPDs, Weeden et al. (1992) reported error rates of 2–4% in segregation analysis and did not exclude rates up to 10% in population studies. Nevertheless, it is important in AFLP population studies to repeat experiments and discard faint fragments from the analysis.

In the present AFLP study the genetic similarity of the analysed hop cultivars was very high. The maximum distance was observed for the powdery mildew-resistant cultivar 'Wye Target' and the land varieties at a level of about 10%. In other AFLP population studies the genetic distance of cultivars was at least twice as high (Maughan et al. 1996; Sharma et al. 1996; Tohme et al. 1996). The high level of genetic similarity observed in this study is in accordance with RAPD assays which resulted in only a few primers differentiating the hop lines (Brady et al. 1996; Pillay and Kenny 1996).

No polymorphism could be detected between the Saazer "noble" hops using 50 additional primer combinations (data not shown). Hence, it is assumed that the varieties 'Saazer', 'Spalter' and 'Tettnanger' are derived from selection breeding in an almost genetically homogenic population. These lines have only small variations in morphological traits and aroma components. Because of the high genetic similarity among these three genotypes, the phenotypic differences may be influenced by a small region of the genome. The cultivar 'Perle', which is a descendant of the female 'Northern Brewer' and the male breeding material from Hüll, was located between the mother line and 'Hallertauer Mittelfrüher'. The two bitter hops 'Wye Target' and 'Hallertauer Magnum' had different positions in our phylogenetic tree, which may be explained by the different breeding origins.

In conclusion, the AFLP technique gives the opportunity of generating sufficient numbers of polymorphic markers in few experiments. Of special importance is that in closely related populations the discrimination of genotypes and the estimation of genetic similarity becomes possible.

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