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Evaluation of AFLP in *Beta*

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Abstract AFLP markers were evaluated for their usefulness in the genetic analysis of sugarbeet and wild *Beta* species. Accessions of ten different sugarbeet breeding lines and five wild beets were screened using 256 primer combinations. Of the 11 309 bands investigated, 96.4% were polymorphic among the accessions. A strong positive correlation was found between the number of polymorphisms and AT content of the selective bases of the primer combinations. Random subsets of primer combinations were used to produce genetic distance trees. Permutation tests showed that, for the wild beets, 500 AFLP bands sufficed to obtain the best topology of the tree with a probability at any given node of more than 99%. Ten times as many bands were necessary to obtain support values of the same order of magnitude for the sugarbeet lines. The reproducibility of AFLP for seven primer combinations was investigated by repeated analysis of all steps from DNA isolation to data scoring. For 5088 comparisons, the overall reproducibility was 97.6%. Robustness to genotyping errors was investigated by including an artificial F_1 (1:1 DNA mixture) of two sugarbeet lines in the screen for polymorphisms. For the 3160 cases of polymorphism between the two lines, 0.2% genotyping errors were found. The general reliability and usefulness of AFLP markers are discussed in relation to the results obtained.

Key words AFLP · *Beta* · Polymorphism · Reproducibility · Sugarbeet

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Introduction

Amplified fragment length polymorphism (AFLP) is a multiplex, polymerase chain reaction (PCR)-based marker system that selectively amplifies a subset of genomic restriction fragments (Zabeau and Vos 1993). Its capacity to detect a large number of loci per assay has led to it being recognised in plant breeding as a highly useful technology for marker-assisted breeding and genotyping applications (Tanksley et al. 1995; Powell et al. 1996). It has been used to study genetic diversity and phylogenetic relationships in a wide range of plant species (Hill et al. 1996; Maughan 1996; Sharma et al. 1996; Tohme et al. 1996; Pakniyat et al. 1997). AFLP markers have been used to produce dense molecular marker maps in such crops as barley (Becker et al. 1995; Powell et al. 1997; Waugh et al. 1997), rice (Maheswaran et al. 1997; Nandi et al. 1997), potato (van Eck et al. 1995; Rouppe van der Voort et al. 1997) and soybean (Keim et al. 1997). In combination with bulked segregant analysis, AFLP technology has been shown to be a powerful tool for the map-based cloning of plant pathogen-resistance genes (Meksem et al. 1995; Thomas et al. 1995; Brigneti et al. 1997; Simons et al. 1997).

In comparison with other multiplex, PCR-based marker systems, AFLP has been found to be a highly reliable and reproducible marker system (Vos et al. 1995; Jones et al. 1997). Several reports on the extensively used random amplified polymorphic DNA (RAPD) system (Williams et al. 1990) have focused on difficulties in obtaining reproducible results (Weeden et al. 1992; Ellsworth et al. 1993; Penner et al. 1993). In particular, RAPD markers have been shown to be unreliable as genetic markers due to the occurrence of competition during PCR amplification (Halldén et al. 1996). Nevertheless, in many applications RAPD markers have been used successfully. Other multiplex marker systems, such as arbitrary primer (AP)-PCR

(Welsh and McClelland 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles et al. 1991) inter-simple sequence repeats (ISSR) (Zietkiewicz et al. 1994) and random amplified microsatellite polymorphism (RAMP) (Matioli and de Brito 1995; Sanchez de la Hoz et al. 1996), are based on principles of PCR amplification similar to those of RAPD. Therefore, although their properties have not been thoroughly investigated, it is likely that these marker systems also have limitations in reproducibility similar to those of the RAPD system. In contrast, AFLP technology combines the reliability of restriction enzyme digestion with stringent PCR conditions for primer annealing (Vos et al. 1995). This has led to the general view that AFLP is the most robust of all the multiplex marker systems currently available. It is surprising, though, that relatively little has been done to investigate the actual reliability of AFLP.

AFLP markers have previously been reported to be useful for genetic mapping in sugarbeet (Schondelmaier et al. 1996). In the study reported here, we investigated their usefulness in fingerprinting, as well as their level of reproducibility and robustness, and some general characteristics of the AFLP system. The results should be applicable to other plant species.

Materials and methods

Plant material and DNA isolation

Ten inbred diploid sugarbeet breeding lines of different origin, representing a wide range of the current germplasm, were selected for the study. In addition, five USDA-ARS accessions of diploid wild beet species representing two sections of the genus *Beta* were analysed. The *Beta* section was represented by two samples of *B. vulgaris* ssp. *maritima*, collected in England and Turkey, respectively, and one sample of *B. patula* collected in the Madeira Islands. The *Procumbentes* section was represented by one sample each of *B. procumbens* and *B. webbiana*, both of unknown origin. DNA from the sugarbeet breeding lines and from the wild beets was isolated from freeze-dried leaves using the CTAB protocol described by Hjerdin et al. (1994).

AFLP analysis

The AFLP analysis was performed essentially as described by Vos et al. (1995). First, 500 ng DNA was digested in 40 μl of 1 × TA buffer (10 m*M* TRIS-acetate, 10 m*M* MgAc, 50 m*M* KAc, 1 m*M* DTT; Advanced Biotechnologies, UK), 2 μg BSA and 5 u each of *Eco*RI (Advanced Biotechnologies) and *Tru*1I (MBI Fermentas, Lithuania). *Eco*RI is referred to in the following as E, and *Tru*1I, an isoschizomer of *Mse*I, as M. Following digestion, 10 μl of ligation solution containing 1 × ligation buffer [40 mM TRIS-HCl (pH 7.8), 10 m*M* $MgCl_2$, 10 m*M* DTT, 0.5 m*M* ATP; MBI Fermentas], 1 U T4 DNA ligase (MBI Fermentas), 0.1 μ*M* E-adapter and 1.0 μ*M* M-adapter, with sequences as described by Vos et al. (1995), was added directly to the DNA digest, incubated, and subsequently diluted ten-fold in 1 × TE buffer. Preamplification by primers having one selective nucleotide each, namely E + 1 and M + 1, was performed in either Perkin-Elmer/Cetus 9600 or MJ Research PTC-100 thermocyclers using the following temperature profile: 20 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The 20 μl of reaction solution contained 5 μl of template DNA, 1 × PCR buffer II [10 m*M* TRIS-HCl (pH 8.3), 50 m*M* KCl; Advanced Biotechnologies], 0.2 m*M* dNTP (Pharmacia, Sweden), 1.5 m*M* $MgCl_2$, 0.4 U *Taq*-polymerase (Advanced Biotechnologies) and 0.3 μ*M* each of (E + A)-primer and (M + C)-primer (DNA Technologies, Denmark). Prior to selective amplification, the (E + 3)-primer was end-labeled in a solution containing 1 × kinase buffer [50 m*M* TRIS-HCl (pH 7.6), 10 m*M* $MgCl_2$, 5 m*M* DTT, 0.1 m*M* spermidine, 0.1 m*M* EDTA; MBI Fermentas], 1.7 μ*M* (E + 3)-primer (DNA Technologies), 0.2 U/μl T4 polynucleotide kinase (MBI Fermentas) and 0.67 kBq/μl γ-[^{33}P]-ATP (Amersham, UK). Selective amplification was performed using the following temperature profile: 12 cycles of 30 s at 94°C, 30 s at 65°C, ramping −0.7°C/cycle to 56°C, 60 s at 72°C; followed by 23 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The 20 μl of reaction solution contained 5 μl preamplified template DNA, 0.5 μl labeled (E + 3)-primer, 1 × PCR buffer II (Advanced Biotechnologies), 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, 0.25 μ*M* (M + 3)-primer (DNA Technologies) and 0.4 U of *Taq*-polymerase (Advanced Biotechnologies). Following amplification, 20 μl of formamide loading buffer (98% formamide, 10 m*M* EDTA, 0.1% each of xylene cyanol and bromophenol blue) was added, and the samples were denatured at 95°C for 3 min. Amplified fragments were separated on 5% polyacryamide gels consisting of 19 : 1 Acrylamide/Bis solution (Labassco), 1 × TBE buffer, 0.10% TEMED and 0.03% APS. A custom-made gel apparatus for 35-cm gels (CBS Scientific Co., USA) was used in all analyses. The gels were pre-run at 110 W for 30 min prior to the loading of 3 μl of sample and were run then at 110 W for approximately 3.5 h. Following electrophoresis, the gels were transferred to 3MM paper, dried on a gel dryer overnight at 80°C and exposed to film for 1–2 days. Each band was scored twice by two different persons.

Analysis of genetic relationships

Genetic relationships among the accessions were analysed in two different ways. For the sugarbeet breeding lines, genetic distances were calculated as $1 - (2X_{i,j}/(X_i + X_j))$, where $X_{i,j}$ is the number of bands found in both lines *i* and *j*, and X_i and X_j are the numbers of bands found in lines *i* and *j*, respectively. This matrix was used then to construct genetic distance trees using the UPGMA method of the PHYLIP programme package (Felsenstein 1993). Phylogenetic trees for the wild beets were created by a parsimony method, the DOLLO subroutine of the DOLLOP programme. The presence and absence of bands were coded as uniquely defined binary characters. Bootstrap and permutation data sets were created by the random selection of loci, with and without replacement, respectively. These data sets were analysed then in the same way as the original data sets, the resulting permutation and bootstrap trees being compared using the CONSENSE programme of the PHYLIP programme package.

Results

Performance and usefulness

In order to investigate the usefulness of AFLP in sugarbeet, we used 256 primer combinations to screen ten different sugarbeet breeding lines, one artificial F_1 of two of the lines (1 : 1 mixture of DNA) and five samples of wild beets representing four different species. The number of scorable bands per primer combination varied from 11 to 116, with an average of 44.3. A total of

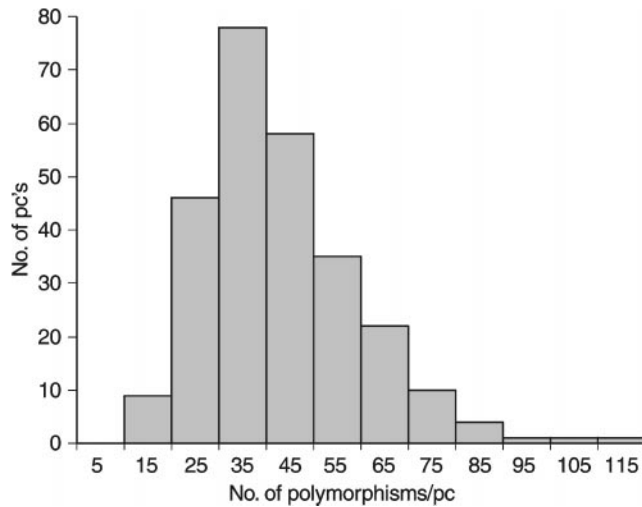

Fig. 1 Distribution of the number of primer combinations (*pc's*) generating different numbers of polymorphisms

11 309 bands in the samples were investigated. Most of the bands (96.4%) were polymorphic between at least two of the samples. The numbers of polymorphic bands per primer combination showed a skewed distribution, some primer combinations detecting twice as many as the average primer combination (Fig. 1). The 10% most polymorphic primer combinations generated 17.5% of the polymorphic bands. A strong positive correlation (Spearman rank = 0.51) was found between the number of bands per primer combination and the overall AT content in the selective bases of the primers (Fig. 2). For each additional A or T, irrespective of position among the selective bases, an average of 8.9 additional bands were detected. This type of correlation has previously been reported in barley (Qi and Lindhout 1997) and can be expected in other plant species as well, plants being generally AT-rich.

The average number of bands and polymorphisms detected per primer combination in pairwise comparisons of different accessions varied markedly (Table 1). The band patterns of the two *Procumbentes* accessions were very similar to each other, with an average of less than 1 polymorphism among the approximately 14 bands per primer combination. For the pairwise comparisons of accessions of section *Beta* the level of polymorphism was much higher, almost 10 polymorphisms being found among 25–30 bands. A comparison of the section *Beta* and section *Procumbentes* accessions revealed a high level of polymorphism, 27 polymorphisms among 32 bands being found. Hence, the sets of AFLP bands detected in sections *Beta* and *Procumbentes* differed almost completely.

A strong positive correlation (Spearman rank = 0.66) with the number of polymorphisms per primer combination in the different pairs of accessions was obtained. A primer combination that detects a larger than average number of polymorphisms between 2 accessions is often highly polymorphic between other accessions. For a set of primer combinations, selected as being the most polymorphic in one pair of accessions, a greater number of polymorphisms was found to be generated in another pair of accessions than for a randomly chosen set of primer combinations (Table 2). The stricter the selection of primer combinations is and the more closely related the pairs of accessions are, the more pronounced this becomes.

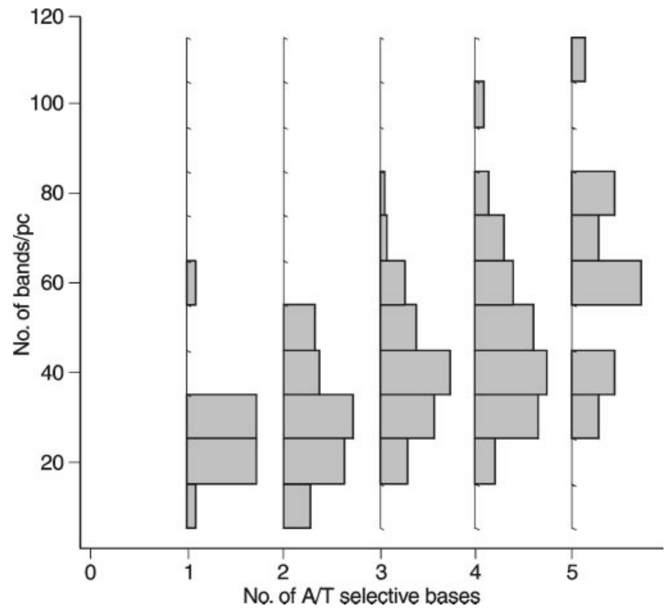

Fig. 2 Correlation between the number of bands per primer combination (*pc*) and the AT content of the selective nucleotides

Table 1 Average numbers of bands and polymorphisms per primer combination (with standard deviations) as detected in pairwise comparisons of accessions

Accession comparisons	Bands	Polymorphisms
Within sugarbeet lines	27.4 ± 12.5	7.4 ± 3.5
Within *B. v.* ssp. *maritima*	26.8 ± 11.7	8.1 ± 4.4
B. v. ssp. *maritima* vs. *B. patula*	24.6 ± 10.9	9.2 ± 4.5
B. webbiana vs. *B. procumbens*	14.2 ± 7.0	0.9 ± 1.2
Beta vs. *Procumbentes*	31.8 ± 13.0	27.1 ± 10.6

Table 2 Ratio of random to selected primer combinations required to detect a given number of polymophisms between a pair of randomly chosen sugarbeet or wild beet accessions. The selected primer combinations represent the 2%, 5%, 10% or 20% most polymorphic primer combinations found in one pair of sugar beet accessions after screening

Accession comparisons	2%	5%	10%	20%
Sugarbeet lines	2.22	1.95	1.77	1.59
Wild beets	1.75	1.62	1.52	1.39

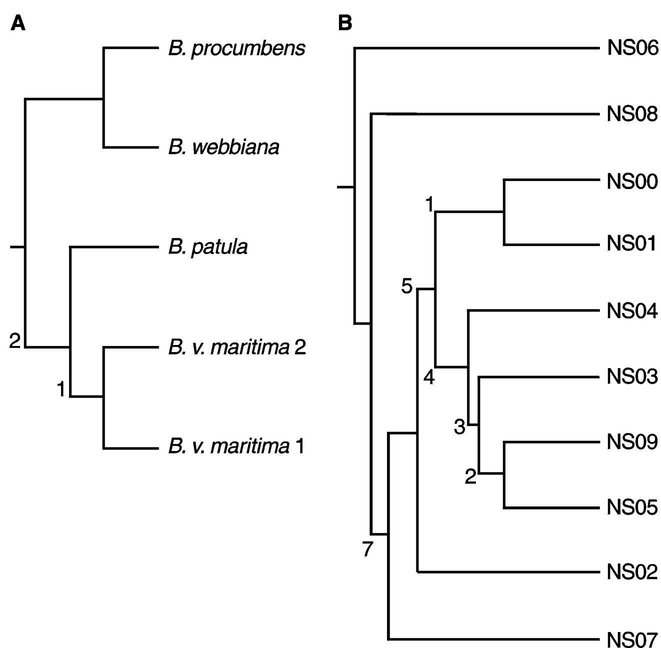

Fig. 3 A Phylogenetic tree of the wild *Beta* accessions with numbered nodes. **B** Genetic distance tree (UPGMA) of the ten sugarbeet breeding lines with numbered nodes

Table 3 Bootstrap values for the trees as based on the complete data set, and results of the permutation analyses for different numbers of bands. The frequencies (%) of congruence with the best trees are shown for the different nodes. The node numbers are the same as in Fig. 3

A) Wild beet tree

Node no.	Bootstrap	Permutations (no. of bands)					
		100	200	500	1000	2000	5000
1	100	84.4	92.9	99.4	100	100	100
2	100	100	100	100	100	100	100

B) Sugarbeet tree

Node no.	Bootstrap	Permutations (no. of bands)					
		100	200	500	1000	2000	5000
1	100	69.6	83.4	95.4	99.7	100	100
2	100	31.8	46.4	65.5	83.1	95.8	100
3	68.9	18.5	28.8	45.3	55.3	59.7	66.2
4	100	24.4	45.2	78.0	95.9	99.6	100
5	99.7	23.9	43.0	65.1	77.6	88.6	99.0
6	100	24.4	44.2	71.6	89.1	98.1	100
7	99.9	23.8	36.3	55.4	73.0	87.4	99.7

The utility of AFLP in assessing genetic relationships was investigated for the wild beets and the sugarbeet breeding lines separately. For the the wild *Beta* species, the relatedness was analysed using a parsimony method (Fig. 3A). The resulting phylogenetic tree corresponds well with the relationships between the different species that are commonly accepted (van Geyt et al. 1990; Jung et al. 1993). All of the nodes in the tree are supported by very high bootstrap values (Table 3A). Permutation analyses were performed to investigate the number of bands required to correctly resolve different nodes in the trees. A specific number of bands were selected randomly and used to create a new tree. This process was repeated 1000 times, for each node the frequency of congruence with the tree based on the complete data set being scored. With only 500 loci, the probability of obtaining any node correctly was more than 99% (Table 3A).

Genetic distances between the sugarbeet breeding lines were calculated, their relationships being visualised by constructing a genetic distance tree using the UPGMA method (Fig. 3B). The tree based on all of the 11 309 bands had very high bootstrap values for most of the nodes (Table 3B) and is assumed, therefore, to accurately reflect the genetic relatedness among the sugarbeet lines. In contrast to the wild beet tree, permutation analyses of the sugarbeet tree showed that for most of the nodes more than 2000 bands were required to obtain a congruence to the topology of the best tree of close to 90% (Table 3B). Node 3 had a very low bootstrap value in the best tree and, thus, has a low frequency of congruence to the best tree in the permutation analysis.

Reproducibility and robustness

To investigate the reproducibility of AFLP, we used seven primer combinations in repeated analyses. All steps, including DNA isolation, restriction-enzyme digestion, ligation of adapters, amplifications and data scoring, were performed twice, independently and on separate occasions. A total of 318 separate bands were amplified and scored for all 16 accessions, the results for the two replicates being compared (Fig. 4). Altogether, 5088 comparisons were evaluated. Three main types of reproducibility errors were found. Firstly, there were 16 errors (0.3%) due to typing mistakes in the digitisation of bands. These errors are attributable entirely to the human factor. Secondly, there were 78 errors (1.5%) due to bands that were clearly resolved in the one experiment being interpreted in the other as a single band. Such errors are attributable to the gel resolution. Thirdly, there were 26 bands (0.5%) found in a specific accession in the one experiment but absent in the other. This type of error can be seen as intrinsic to the AFLP protocol employed.

In many applications, such as mapping for example, a genetic interpretation of the AFLP bands is needed. The simplest interpretation is that the presence and absence of a band corresponds to there being two different alleles at a single locus. If two or more loci

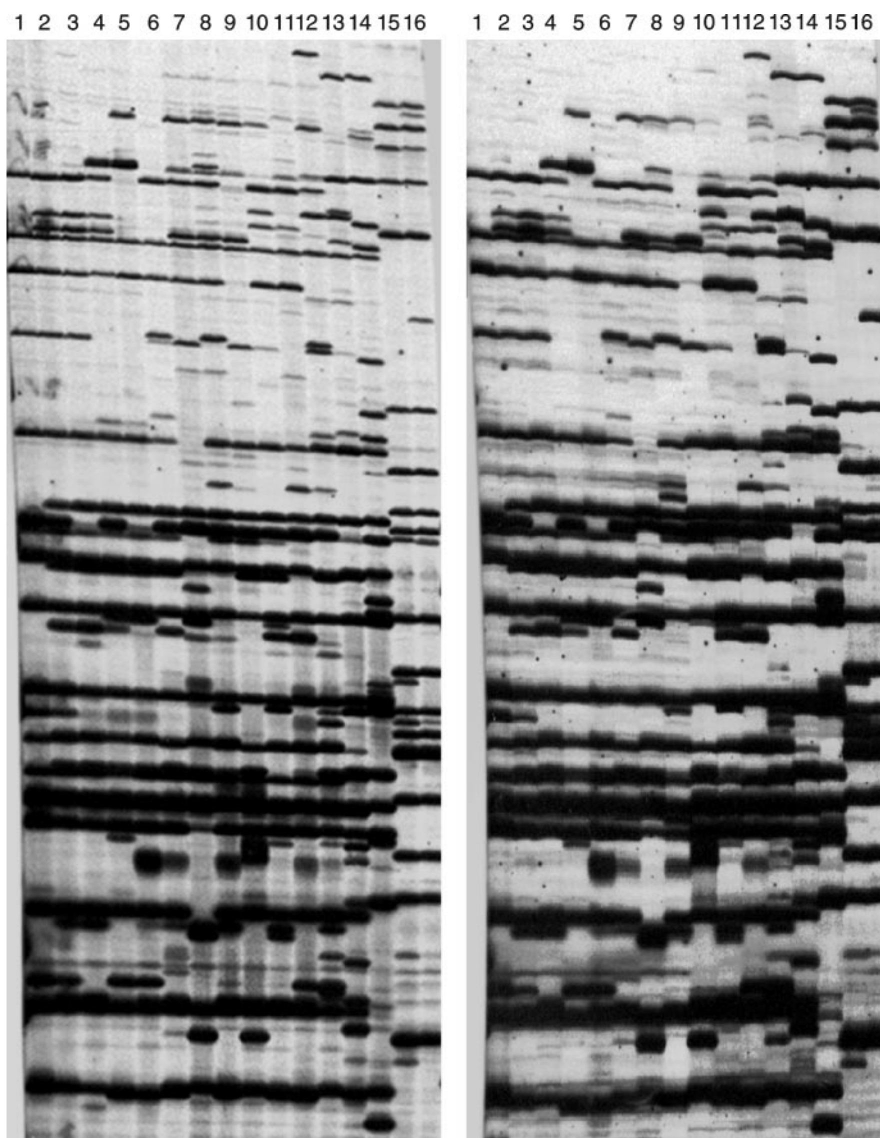

Fig. 4 Example of the reproducibility of AFLP markers for one primer combination and the 16 samples. *Lanes 1* and *3–11* represent the ten sugarbeet breeding lines, *lane 2* represents an artificial F_1 (1:1 DNA mixture) of the lines in *lanes 1* and *3*, *lanes 12* and *13* represent two samples of *B. vulgaris* ssp. *maritima*, *lane 14* a sample of *B. patula*, *lane 15* a sample of *B. webbiana*, *lane 16* a sample of *B. procumbens*

were represented by bands of the same size, this would lead to an erroneous genetic interpretation. To investigate the occurrence of this type of error, we preamplified two genotypes using an (E+1)-primer and an (M+2)-primer. These samples were selectively amplified with eight combinations of (E+3)- and (M+3)-primers. In parallel, the samples were selectively amplified with (E+3)- and either (1) (M+4)-primers having either A, C, T or G in the fourth position or (2) a mixture of the four different (M+4)-primers, referred to as the (M+4)-mixture. In all cases in which a band is amplified by the (E+3)/(M+3) primer combination, the same band is also expected for the (E+3)/(M+4)-mixture primer combination and for one of the (E+3)/(M+4) primer combinations. The amplification of a band by more than one of the (E+3)/(M+4) primer combinations is most easily explained by the occurrence of similar-sized bands from different loci. Of the 456 bands from the (E+3)/(M+3) amplification that were investigated, a total of 396 were present in the (E+3)/(M+4)-mixture amplification and in only one of the (E+3)/(M+4) amplifications (Table 4). The remaining 60 bands (13.2%) were present in the (E+3)/(M+4)-mixture and in more than one of the E+3/M+4 amplifications.

Another type of distortion that can occur in AFLP reactions is the unspecific annealing, mismatch, of the selective bases, leading to the undesired amplification of extra bands. We investigated the occurrence of mismatches using all combinations of (E+A)- or (E+AC)-primer and (M+C)- or (M+CA)-primer for the preamplification of one genotype (Table 5). Selective amplifications were performed with eight (E+3)/(M+3) primer combinations. If no mismatches occurred, the same band pattern would be expected for a given primer combination, irrespective of preamplification

Table 4 Analysis of the number of loci per band. The presence and absence of the band is shown for selective amplification of one genotype using: (1) an (E + 3)/(M + 3) primer combination, (2) an (E + 3)/(M + 4)-mixture primer combination and (3) an (E + 3)/(M + 4) primer combination with an A, C, G or T in the fourth position, respectively. Preamplification of the genotype was performed using an (E + 1)/(M + 2) primer combination

M+3	M+4 mix	M+4 A	M+4 C	M+4 G	M+4 T	Number of occurrences
–	–	–				120
–	–		–			81
–	–			–		87
–	–				–	108
–	–	–	–			6
–	–	–		–		11
–	–	–			–	5
–	–		–	–		9
–	–		–		–	19
–	–			–	–	9
–	–	–	–	–		1

Table 5 Analysis of the occurrence of mismatches. The presence and absence of bands is shown for different numbers of selective bases during preamplification. Selective amplification of the genotype was performed using eight (E + 3)/(M + 3) primer combinations. Bands that are absent in the (E + A)/(M + C) preamplification but present in any other are likely resulting from mismatches

E + A M + C	E + A M + CA	E + AC M + C	E + AC M + CA	Number of occurrences
–	–	–	–	182
	–		–	87
		–	–	40
–		–		10
			–	4
	–	–	–	3
		–		3

conditions. In contrast, if mismatches occurred, extra bands would be expected for some preamplification conditions. A total of 329 bands were scored, 182 (55.3%) being present in all samples. In 87 cases (26.4%), extra bands were found in both the (E + 1)/(M + 2) and the (E + 2)/(M + 2) amplifications. Assuming that the 3′-ultimate base of a primer is less likely to mismatch to the target than any of the other selective bases (Vos et al. 1995), such cases appear to represent a mismatch in the penultimate selective base of the (M + 2)-primer during preamplification. In 40 cases (12.2%), bands were found in both the (E + 2)/(M + 1) and the (E + 2)/(M + 2) amplifications. These situations are thus likely to represent a mismatch in the penultimate selective base of the (E + 2)-primer. Ten cases (3.0%) of bands only occurring in the (E + 1)/(M + 1) and (E + 2)/(M + 1) amplifications appear to be most easily explained by a mismatch in the M-primer during selective amplification. Thus, for selective amplification with an (E + 3)/(M + 3) primer combination, the lowest frequency of mismatch occurs at an (E + 1)/(M + 1) preamplification.

The phenomenon of competition in PCR has been shown in RAPD analysis to result in a considerable number of errors (Halldén et al. 1996). The occurrence of errors in AFLP analysis due to competition was investigated by including in the analyses a 1 : 1 mixture of DNA from two of the inbred (S_3 and S_4, respectively) sugarbeet lines, representing an artificial F_1. A total of 3160 situations in which a band was polymorphic between the inbred lines, and was thus also expected in the artificial F_1, were evaluated. In 3154 (99.8%) of the cases the band expected was also present in the artificial F_1.

Discussion

Due to the large number of loci that are detected per AFLP assay, primer combinations are often chosen more or less randomly, obviating the need for an expensive and time-consuming screen for polymorphisms. However, the extra work of performing a screen in order to identify the most polymorphic primer combinations is to be compared with the estimated labour of using randomly chosen primer combinations in the subsequent analyses. If AFLPs are to be used in several different applications or in routine analyses in a certain crop, a preceding screen for polymorphisms may reduce the subsequent labour markedly. We found there to be an uneven distribution in the number of polymorphisms per primer combination, with a small number of highly polymorphic primer combinations revealing a relatively large proportion of the polymorphisms. This clearly demonstrates the value of a preceding screen to identify polymorphic primer combinations. Consider the following AFLP mapping project: Two parental lines are screened for polymorphisms using 256 primer combinations. Mapping is performed in a population of 200 individuals. Given the distribution of polymorphisms per primer combination in this study (Fig. 1), the total number of AFLP reactions needed for the parental screen and the mapping of 50 markers using the most polymorphic primer combinations is similar to the number of reactions needed if randomly selected primer combinations are used. For a greater number of markers or individuals, the total number of AFLP reactions needed will be larger using random primer combinations as compared to performing a parental screen and using selected ones.

The positive correlations between different genotype comparisons in the numbers of polymorphisms per primer combination also indicates that a screen for polymorphic primer combinations prior to fingerprinting is advantageous. For low numbers of selected primer combinations, more than twice the number of polymorphisms was obtained as compared to the use of random primer combinations.

In fingerprinting, the obvious advantages of AFLP are the robustness and high degree of multiplexity, whereas one drawback is the difficulty in scoring markers codominantly. We investigated the usefulness of AFLP in fingerprinting by separate analyses of the 5 samples of wild beets and the ten sugarbeet lines. Genetic relationships were visualised in terms of phylogenetic and genetic distance trees, respectively. For the different wild *Beta* species, the results corresponded to the existing phylogenetic tree perfectly (van Geyt et al. 1990; Jung et al. 1993), and the phylogeny was resolved using a small number of primer combinations. In contrast, complete resolution of the relatedness between the sugarbeet lines was not obtained, even when all the AFLP markers ($>11\,000$) were applied. In interbred materials the genealogies for different genomic regions differ, and a single true phylogenetic tree does not exist. This explains the rather low bootstrap values obtained for the sugarbeet tree. Thus, the biological interpretation of genetic distance trees based on random markers from the entire genome are unclear. Given the existence of a high-density map, an alternative approach could be to employ map-based fingerprinting. Genetic distance trees are then constructed for smaller genomic regions, possibly resulting in less complex and more robust estimates.

The high degree of reproducibility of AFLP markers between repeated experiments has been shown in this study and among different laboratories by Jones et al. (1997). In addition, the lack of errors due to competition (0.2%) in the PCR amplification of AFLP markers is remarkably different from the 14% reported previously for RAPD markers in *Brassica* (Halldén et al. 1996). Under the AFLP conditions employed here, we have shown that mispriming occurs during preamplification and is manifested after selective amplification. This is somewhat contradictory to the results of Vos et al. (1995), which showed that selectivity is maintained for the two 3′-most nucleotides during amplification in yeast. However, for a given preamplification regime, especially with (E+1)/(M+1)-primers, robust results are obtained, which is reflected in the high reproducibility. Thus, mispriming is a minor problem in AFLP analysis.

Another, but more serious type of problem is the occurrence of overlapping bands from different loci, which causes errors in the genetic interpretation of the bands. In our study 13% of the bands that were amplified for a specific 3′-ultimate selective base were also amplified with another 3′-ultimate base. There are three possible explanations for this: (1) mismatch in the 3′-ultimate base during selective amplification, (2) insufficient gel resolution of bands from different loci and (3) the presence of identically sized, or identically migrating, bands from different loci. The first explanation can not be excluded, but we have shown that the frequency of bands resulting from mismatches during selective amplification is rather low (3.0%). In addition, it is assumed to be more likely that a mismatch would occur in the 3′-penultimate base rather than in the 3′-ultimate base. We therefore believe that mismatch does not greatly influence the observed error rate. The second explanation reflects a source of error due to the specific AFLP system employed. A solution to this problem could be to use gels of better resolution, through their being more dense or the runs being longer. However, this solution might interfere with the number of samples it is possible to analyse per day. We have chosen to favour high throughput by using an AFLP system compatible with the 96-well format, one that allows thousands of samples per week to be analysed. In contrast, the third explanation reflects an inherent feature of the AFLP system which would be very difficult to prevent. One way to reduce the effect of identically sized bands would be to increase the number of selective bases. This would lead simultaneously, however, to a decrease in the overall number of amplified bands, reducing the efficiency of the AFLP system. In analysing genetic variation, the occurrence of overlapping bands from different loci results in a downward bias in estimations of variation, accessions appearing to be more closely related than they actually are. However, since it is relative rather than absolute genetic distances which are the most important in establishing genetic relationships, the bias is not expected to cause any serious distortion. In genetic mapping, the misinterpretation will most likely be manifested as a skewed segregation of the marker band, which should lead to exclusion of the band. Hence, linkage analysis is not seriously affected, although the number of useful markers decreases.

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References

Becker J, Vos P, Kuiper M, Salamini F, Heun M (1995) Combined mapping of AFLP and RFLP markers in barley. Mol Gen Genet 249:65–73

Brigneti G, Garcia-Mas J, Baulcombe DC (1997) Molecular mapping of the potato virus Y resistance gene Ry_{sto} in potato. Theor Appl Genet 94:198–203

Caetano-Anolles G, Bassam BJ, Gresshoff PM (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology 9:553–556

Eck HJ van, Rouppe van der Voort J, Draaistra J, van Zandvoort P, van Enckevort E, Segers B, Peleman J, Jacobsen E, Helder J, Bakker J (1995) The inheritance and chromosomal localization of AFLP markers in a non-inbred potato offspring. Mol Breed 1:397–410

Ellsworth DL, Rittenhouse KD, Honeycutt RL (1993) Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques 14:214–217

Felsenstein, J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, Wash.

Halldén C, Hansen M, Nilsson NO, Hjerdin A, Säll T (1996) Competition as a source of errors in RAPD analysis. Theor Appl Genet 93 : 1185–1192

Hill M, Witsenboer H, Zabeau M, Vos P, Kesseli R, Michelmore R (1996) PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. Theor Appl Genet 93 : 1202–1210

Hjerdin A, Säll T, Tuvesson S, Halldén C (1994) RFLP markers in the genus *Beta*: Characterization of DNA sequences from a *Beta vulgaris* library. Genetica 92 : 91–99

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol Breed 3 : 381–390

Jung C, Pillen K, Frese L, Fähr S, Melchinger AE (1993) Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". Theor Appl Genet 86 : 449–457

Keim P, Schupp JM, Travis SE, Clayton K, Zhu T, Shi L, Ferreira A, Webb DM (1997) A high-density soybean genetic map based on AFLP markers. Crop Sci 37 : 537–543

Maheswaran M, Subudhi PK, Nandi S, Xu JC, Parco A, Yang DC, Huang N (1997) Polymorphism, distribution and segregation of AFLP markers in a doubled haploid rice population. Theor Appl Genet 94 : 39–45

Matioli SR, de Brito RA (1995) Obtaining genetic markers by using double-stringency PCR with microsatellites and arbitrary primers. BioTechniques 19 : 752–758

Maughan PJ, Saghai Maroof MA, Buss GR, Huestis GM (1996) Amplified fragment length polymorphism (AFLP) in soybean: Species diversity, inheritance, and near-isogenic line analysis. Theor Appl Genet 93 : 392–401

Meksem K, Leister D, Peleman J, Zabeau M, Salamini F, Gebhardt C (1995) A high resolution map of the vicinity of the *R1* locus on chromosome V of potato based on RFLP and AFLP markers. Mol Gen Genet 249 : 74–81

Nandi S, Subudhi PK, Senadhira D, Manigbas NL, Sen-Mandi S, Huang N (1997) Mapping QTLs for submergence tolerance in rice by AFLP analysis and selective genotyping. Mol Gen Genet 255 : 1–8

Pakniyat H, Powell W, Baird E, Handley LL, Robinson D, Scrimgeour CM, Nevo E, Hackett CA, Caligari PDS, Forster BP (1997) AFLP variation in wild barley (*Hordeum vulgare* C. Koch) with reference to salt tolerance and associated ecogeography. Genome 40 : 332–341

Penner GA, Bush A, Wise R, Kim W, Domier L, Kasha K, Laroche A, Scoles G, Molnar SJ, Fedak G (1993) Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Methods Applic 2 : 341–345

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 2 : 225–238

Powell W, Thomas WTB, Baird E, Lawrence P, Booth A, Harrower B, McNicol JW, Waugh R (1997) Analysis of quantitative traits in barley by the use of amplified fragment length polymorphisms. Heredity 79 : 48–59

Qi X, Lindhout P (1997) Development of AFLP markers in barley. Mol Gen Genet 254 : 330–336

Rouppe van der Voort JNAM, van Zandvoort P, van Eck HJ, Folkertsma RT, Hutten RCB, Draaistra J, Gommers FJ, Jacobsen E, Helder J, Bakker J (1997) Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes. Mol Gen Genet 255 : 438–447

Schondelmaier J, Steinrücken G, Jung C (1996) Integration of AFLP markers into a linkge map of sugar beet (*Beta vulgaris* L.). Plant Breed 115 : 23–237

Sharma SK, Knox MR, Ellis THN (1996) AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. Theor Appl Genet 93 : 751–758

Sánchez de la Hoz MP, Dávila JA, Loarce Y, Ferrer E (1996) Simple sequence repeat primers used in polymerase chain reaction amplifications to study genetic diversity in barley. Genome 39 : 112–117

Simons G, van der Lee T, Diergaarde P, van Daelen R, Groenendijk J, Frijters A, Büschges R, Hollricher K, Töpsch S, Schulze-Lefert P, Salamini F, Zabeau M, Vos P (1997) AFLP-based fine mapping of the *Mlo* gene to a 30-kb DNA segment of the barley genome. Genomics 44 : 61–70

Tanksley SD, Ganal MW, Martin GB (1995) Chromosome landing: a paradigm in map-based gene cloning in plants with large genomes. Trends Genet 11 : 63–68

Thomas CM, Vos P, Zabeau M, Jones DA, Norcott KA, Chadwick BP, Jones JDG (1995) Identification of amplified restriction fragment polymorphism (AFLP) markers tightly linked to the tomato *Cf-9* gene for resistance to *Cladiosporum fulvum*. Plant J 8 : 785–794

Tohme J, Orlando Gonzalez D, Beebe S, Duque MC (1996) AFLP analysis of gene pools of a wild bean core collection. Crop Sci 36 : 1375–1384

Van Geyt JPC, Lange W, Oleo M, DeBock ThSM (1990) Natural variation within the genus *Beta* and its possible use for breeding sugar beet: a review. Euphytica 49 : 57–76

Vos P, Hogers R, Bleeker M, Reijans M, van der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res 23 : 4407–4414

Waugh R, Bonar N, Baird E, Thomas B, Graner A, Hayes P, Powell W (1997) Homology of AFLP products in three mapping populations of barley. Mol Gen Genet 255: 311–321

Weeden NF, Timmerman GM, Hemmat M, Kneen BE, Lodhi MA (1992) Inheritance and reliability of RAPD markers. In: Hoisington D, McNab A (eds) Proc Symp Appl RAPD Technol Plant Breed. Crop Sci Soc Am, Minneapolis, pp 12–17

Welsh J, McClelland J (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18 : 7213–7218

Williams JGK, Kubelik AR, Livak KJ, Rafalski A, Tingey SV (1990) DNA polymorphisms amplified by arbitary primer are useful as genetic markers. Nucleic Acids Res 18: 6531–6535

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Appl 92402629.7

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20 : 176–183