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Development of AFLP markers in barley

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Abstract To investigate the application of amplified fragment length polymorphism (AFLP) markers in barley, 96 primer combinations were used to generate AFLP patterns with two barley lines, L94 and Vada. With seven primer combinations, only a few intense bands were obtained, probably derived from repeated sequences. With the majority of the remaining 89 primer combinations, on average about 120 amplification products were generated, and the polymorphism rate between the two lines was generally over 18%. Based on the number of amplified products and the polymorphism rate, the 48 best primer combinations were selected and tested on 16 barley lines, again including L94 and Vada. Using a subset of 24 primer combinations 2188 clearly visible bands within the range from 80 to 510 bp were generated; 55% of these showed some degree of polymorphism among the 16 lines. L94 versus Vada showed the highest polymorphism rate (29%) and Proctor versus Nudinka yielded the lowest (12%). The polymorphism rates per primer combination showed little dependence on the barley lines used. Hence the most efficient and informative primer combinations identified for a given pair of lines turned out to be highly efficient when applied to others. Generally, more than 100 common markers (possibly locus specific) among populations or crosses were easily identified by comparing 48 AFLP profiles of the parent lines. The existence of such a large number of markers common to populations will facilitate the merging of molecular marker data and other genetic data into one integrated genetic map of barley.

Key words *Hordeum vulgare* · AFLP markers · Genetic variation

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Introduction

The use of restriction fragment length polymorphisms (RFLPs) as DNA markers to construct genetic maps was first proposed by Botstein et al. (1980). Since then, various DNA markers have been developed and applied in many organisms. In plants, DNA markers have been used for genetic and genome studies, and more recently, to facilitate gene cloning and practical breeding. RFLP markers have been particularly suitable for genetic map construction and synteny studies among crop species. The comparison of RFLP maps of several cereal species has identified homologous chromosome segments in many different species (Bennetzen and Freeling 1993). This synteny should facilitate the isolation of genes from species with a large genome, such as wheat, by map-based cloning of the corresponding homologous segments from species with small genomes, like rice (Kilian et al. 1995). In barley (*Hordeum vulgare*), the first molecular marker linkage map (chromosome 6) was generated based on RFLPs by Kleinhofs et al. (1988). So far, more than 1000 molecular markers, predominantly RFLPs, have been mapped on the barley genome. Recently, the genetic linkage maps of four doubled haploid populations: Proctor × Nudinka (Heun et al. 1991), Igri × Franka (Graner et al. 1991), Steptoe × Morex (Kleinhofs et al. 1993) and Harrington × TR306 (Kasha and Kleinhofs 1994) have been integrated into one composite map comprising 880 marker loci (Qi et al. 1996).

The RFLP technique requires a relatively large amount of DNA for optimal results from Southern hybridisations. Due to its large genome size (1C = 5.1×10^9 bp; Bennett and Leitch 1995), and the relatively low variation within the barley species, RFLP analyses are labour-intensive and time-consuming. Consequently, other molecular markers, predominantly based on PCR methods, like RAPDs (Welsh and McClelland, 1990; Williams et al. 1990), have also been identified in barley (Kleinhofs et al. 1993). However,

poor reproducibility and population specificity have limited the use of RAPDs for genetic studies. In addition, microsatellites or simple sequence repeats (SSR) have been investigated as DNA markers. Saghai-Maroof et al. (1994) identified 71 alleles among 207 accessions of wild and cultivated barley accessions by using only four microsatellite primer pairs. Becker and Heun (1995) identified 32 alleles among 11 lines by using 15 primer pairs and mapped five microsatellite markers on three barley chromosomes. With large numbers of alleles at one locus, microsatellite markers are very suitable as universal, locus-specific markers over populations. However, development of a sufficient number of microsatellite markers to cover the entire barley genome is still in its infancy. In a collaborative effort, several European research groups aim to develop another 200 microsatellite markers for the barley genome (Waugh 1995).

More recently, a novel DNA fingerprinting technique called AFLP has been developed (Zabeau and Vos 1993; Vos et al. 1995). The technology is based on the amplification of selected restriction fragments of a total genomic digest by PCR, and separation of labelled amplified products by denaturing polyacrylamide gel electrophoresis. A great advantage of the AFLP technique is that it allows simultaneous identification of a large number of amplification products. One hundred and eighteen AFLP markers have already been mapped on the barley genome by using the Proctor × Nudinka doubled haploid population which had previously been used for construction of an RFLP map (Heun et al. 1991; Becker et al. 1995). In a project to map the genes for partial resistance to barley leaf rust (*Puccina hordei*), we also chose AFLP markers as they allow the construction of a high-density genetic map in the most efficient way. In the present study, the variation in AFLP patterns within the barley species was investigated with a large number of primer combinations. Firstly, AFLP fingerprints of two barley lines were obtained using 96 primer combinations, and, secondly, 16 representative barley lines were used to generate AFLP profiles by using 48 primer combinations. These results may facilitate the wider use of AFLPs for extended genetic studies in barley.

Materials and methods

Plant materials

Sixteen barley lines, Harrington, TR306, Steptoe, Morex, Igri, Franka, Proctor, Nudinka, Apex, Prisma, C92, C118, C123, L94, Vada and 116-5, which represent a wide range of the genetic variation in barley (*H. vulgare*), were used in the present research. The first eight lines have been used to generate four doubled haploid populations for the construction of four individual RFLP maps (Heun et al. 1991; Graner et al. 1991; Kleinhofs et al. 1993; Kasha and Kleinhofs 1994) and two integrated maps (Langridge et al. 1995, Qi et al. 1996). Apex and Prisma are Dutch two-rowed spring barley cultivars with medium and good malting quality, respectively. C92, C118 and C123 are partially resistant to *P. hordei* (Niks 1982) and are derived from the barley composite XXI (Suneson and Wiebe 1962), which was based on intercrossing of 6200 cultivars and lines. L94 is a line from an Ethiopian land race and is extremely susceptible to *P. hordei*. Vada is a commercial cultivar from the Department of Plant Breeding, Wageningen Agricultural University, and has a high level of partial resistance. 116-5 is derived from Cebada Capa (of North African origin) × L94 and selected for a high level of partial resistance to *P. hordei*.

The AFLP protocol

DNA was extracted from leaf tissue, frozen in liquid nitrogen, of two-week-old seedlings according to the CTAB protocol published by Van der Beek et al. (1992).

The AFLP technique has been described by Zabeau and Vos (1993) and Vos et al. (1995). The procedure was performed essentially as described by Van Eck et al. (1995) for potato, with some minor modifications.

For template preparation, the selection of biotinylated DNA restriction fragments was omitted. After the restriction-ligation reaction, the restriction enzymes and ligase were denatured at 60° C for 10 min. Subsequently, products were diluted ten-fold in $T_{0.1}E$ buffer and stored at 4° C for pre-amplification.

To obtain good separation of amplified DNA fragments, buffer gradient electrophoresis was conducted with 1 × TBE (100 mM Tris, 100 mM Boric acid, 2 mM EDTA, pH 8.0) in the cathode buffer (−) and 1 × TBE plus 0.5 M sodium acetate in the anode buffer (+).

Adapters, *Mse*I site primers and *Eco*RI site primers used are listed in Table 1.

Table 1 Lists of primers and adapters

Primers/adapters		Sequences[a]
*Mse*I adapter		5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTC AT-5′
M00 (universal primer)		GATGAGTCCTGAG TAA
*Mse*I + 1 primer	M02	M00 + C
*Mse*I + 3 primers	M47	M00 + CAA
	M48	M00 + CAC
	M49	M00 + CAG
	M50	M00 + CAT
	M51	M00 + CCA
	M54	M00 + CCT
	M55	M00 + CGA
	M58	M00 + CGT
	M59	M00 + CTA
	M60	M00 + CTC
	M61	M00 + CTG
	M62	M00 + CTT
*Eco*RI adapter		5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGCATGG TTAA-5′
E00 (universal primer)		GACTGCGTACC AATTC
*Eco*RI + primer	E01	E00 + A
*Eco*RI + 3 primers	E32	E00 + AAC
	E33	E00 + AAG
	E35	E00 + ACA
	E38	E00 + ACT
	E39	E00 + AGA
	E42	E00 + AGT
	E44	E00 + ATC
	E45	E00 + ATG

[a] DNA sequences are always given in the 5′ to 3′ orientation unless indicated otherwise

Data evaluation and nomenclature

The AFLP amplification products were designated according to the restriction enzymes and the primer combination used, and their sizes estimated with reference to the SequaMark 10 base ladder (Research Genetics, Huntsville, ala.).

Results and discussion

AFLP pattern of two barley lines with 96 primer combinations

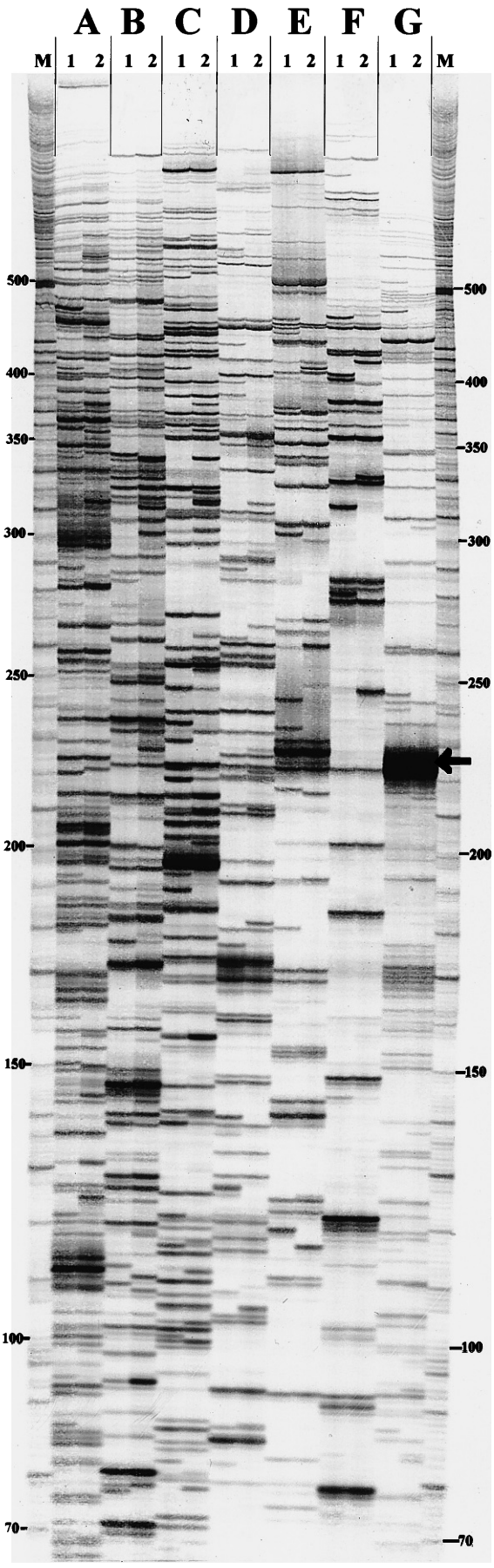
In the present study, eight *Eco*RI primers and twelve *Mse*I primers, each with three selective bases, were used to generate AFLP fingerprints for two barley lines, L94 and Vada. With the majority of the 96 primer combinations, about one hundred fragments were obtained from each barley line. However, several primer combinations produced rather complex profiles which comprised up to 150 bands (Table 2). With seven primer combinations, namely E32M59, E33M49, E35M51, E38M49, E39M62, E42M49 and E44M51, the majority of the labelled primer was incorporated into a single fragment and other fragments appeared as very faint bands (e.g. E35M51 in Fig. 1). Therefore these seven primer combinations are not useful for genetic studies in barley. The appearance of single intense bands is probably due to a high copy number of one particular DNA restriction fragment in the template (Vos et al. 1995). The large genome size of barley ($1C = 5.1 \times 10^9$ bp; Bennett and Leitch 1995) could well harbour a high proportion of repetitive sequences. Indeed, relatively intense bands were detected by using other primer combinations as well, but they did not obscure other fragments.

The total number of bands generated by the different primer combinations revealed a large range of variation, from about 50 bands for E42M58 to 180 for E33M50 (Fig. 1 and Table 2). The range in number of bands showed more variation with the 12 *Mse*I primers (67 to 142 bands) than with the 8 *Eco*RI primers (100 to 130 bands). With M58 on average about 70 visible bands were generated, whereas about 150 bands were produced with M47 and with M50. The three selective nucleotides of primer M47 and M50 are CAA and CAT, respectively (Table 1). Most plant DNAs are AT-rich and if the genome size is large, as in barley, it is better to use AT-poor primers with which fewer bands will be amplified. The 20 primers (12 *Mse*I primers and 8 *Eco*RI primers) used in the present research were in fact AT-poor. As primers more variation in AFLP patterns was observed with *Mse*I than with *Eco*RI primers, the selection of the most informative *Mse*I primers is more critical.

Table 2 Evaluation of 96 primer combinations based on two barley lines L94 and Vada

Polymorphism rates	Number of bands			
	< 90	90–120	120–150	> = 150
< 18%	Fair E45M58	Fair E32M49, E32M54 E33M51, E35M60 E38M62, E39M51 E42M59, E45M60	Fair E33M62, E38M50 E44M54, E44M60 E44N62, E45M48 E45M50	Poor E32M47, E44M47 E44M50, E45M62
18%–23%	Good E32M58, E38M58	Good E32M51, E32M60 E33M59, E38M47 E38M48, E38M51 E38M60, E39M47 E39M49, E39M59 E39M60, E42M62 E44M61	Fair E32M50, E35M62 E39M50, E44M48 E44M49, E45M51 E45M54, E45M59	Poor E33M47, E33M48 E33M50, E35M47
23%–28%	Good E32M55, E39M55 E42M60, E42M61 E44M58	Good E32M62, E35M49 E38M59, E39M48 E39M54, E42M47 E42M50, E45M49 E45M61	Good E32M48, E33M60 E35M54, E35M59 E44M55, E44M59	Fair E35M50, E45M47
> 28%	Excellent E33M58, E35M55 E35M58, E38M55 E38M61, E39M58 E42M51, E42M54 E42M55, E42M58	Excellent E32M61, E33M55 E33M61, E35M48 E35M61, E38M54 E39M61, E42M48 E45M55	Good E33M54	Fair –

Evaluations are based on polymorphism rates and number of bands. E32M59, E33M49, E35M51, E38M49, E39M62, E42M49, E44M51 were not included due to their excessively amplified single fragment



The polymorphism rates and total number of bands with the other 89 primer combinations were evaluated per primer combination (Table 2). The most useful primer combinations have a high polymorphism rate and generate a reasonable number of total bands, that are clearly visible. Based on our results, 48 primer combinations were selected and used to generate AFLP profiles for 16 representative barley lines.

AFLP profiles of sixteen selected barley lines

The AFLP profiles of 16 barley lines were analysed to estimate the sizes of clearly visible bands. Band names were assigned and indicated on images, which are available in GrainGenes on the Internet (http://grain.jouy.inra.fr/ggpages/).

The sizes of fragments generated with 96 primer combinations ranged from about 70 bp to 1 kb (Figs. 1, 2), but most fragments were smaller than 500 bp. Using buffer gradient electrophoresis the larger fragments up to 500 bp were well separated, while only limited information from the smaller bands was lost. Fragments larger than 500 bp were not well separated and were beyond the size marker range used. Consequently, their sizes were estimated by extrapolation and hence are not very accurate. In addition, larger fragments normally gave weaker signals and were more dependent on the quality of templates. So only bands of 80–510 bp were taken into account as summarised in Table 3. Twenty-four primer combinations were selected to study polymorphism rates among 16 barley lines. These primer combinations were recommended by KeyGene, Wageningen or were chosen on the basis of our initial survey (see Table 2).

Figure 2 is an example of the AFLP profiles generated, obtained by using the primer combination E33M61: within the size range of 80 to 510 bp, 106 AFLP bands were observed among the 16 barley lines; 35 were present in all 16 lines and the presence of other 71 bands varied over the 16 lines giving 67% polymorphism rate (Table 3). The large number of bands and the high polymorphism rate among the 16 barley lines indicated that AFLP is an extremely efficient technique for marker generation in barley. A parallel study of genetic relationships in barley showed that with a single primer combination sufficient DNA markers could be screened to unambiguously discriminate between 29 related barley lines (not shown). In the present day, data from 24 primer combinations were evaluated to illustrate the usefulness of AFLP markers in barley.

Fig. 1 Variation in AFLP patterns between two barley lines. AFLP patterns obtained with the seven primer combinations E33M47, E33M48, E33M59, E35M61, E35M55, E35M58 and E35M51 (A–G, respectively). M is a marker lane with 10 bp DNA size markers, 1 and 2 represent L94 and Vada. A–F show AFLP patterns ranging from complex to simple. G is a primer combination showing one very intense fragment (indicated by the *arrow*) and many faint bands

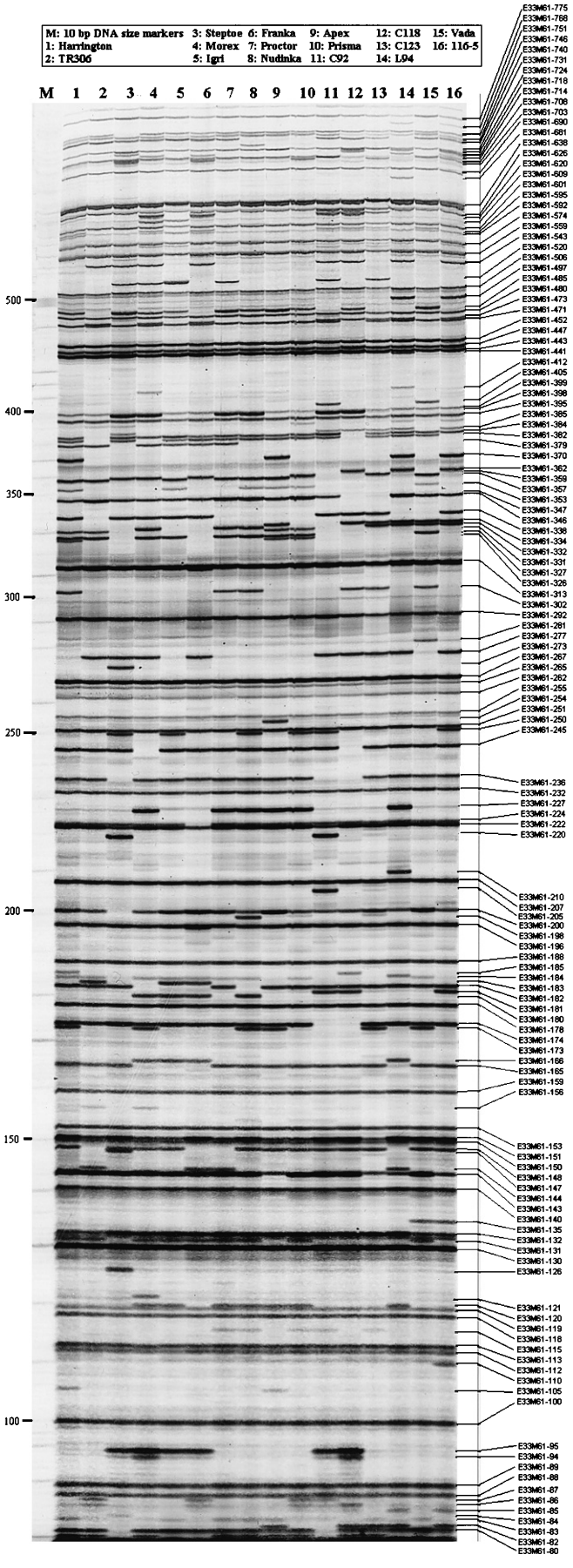


Fig. 2 AFLP fingerprints of 16 barley lines generated with E33M61. 1–16 indicate the barley lines listed in the box at the top. M is a marker lane with 10 bp DNA size markers. All clearly visible bands are connected by lines to their corresponding designations on the right of the panel. Band sizes over 500 bp were estimated by extrapolation

Variation in polymorphism rates

Pair-wise comparisons of six parent pairs, that have been used for generating RFLP maps, showed quite different polymorphism rates (Table 3). The average polymorphism rate per set of two barley lines over 24 primer combinations ranged from only 12.2% between Proctor and Nudinka to 29% between L94 and Vada. Obviously, the genetic distance between L94, a line from an Ethiopialandrace, and the European cultivar Vada was larger. Proctor and Nudinka are closer related because both are two-rowed spring barley cultivars, bred in England and Germany, respectively. Consequently, these high polymorphism rates between L94 and Vada should facilitate the construction of a high-density AFLP map. By using a large number of AFLP markers it should be possible to fill in some of the gaps in the integrated RFLP map (Qi et al. 1996).

The average polymorphism rate per primer combination over 6 parent pairs was 20%, with a range from 13% for E38M51 to 28% for E33M61. Between L94 and Vada, a 41% polymorphism rate was observed with primer combination E42M48 but only 18% with E45M58. The ranking of primer combinations based on polymorphism rates was only weakly dependent upon the barley lines used for comparison. Thus, the most efficient and informative primer combinations identified for a given set of barley lines are likely to be most efficient when applied to other lines also.

A very similar AFLP polymorphism rate (11.3%) was observed between Proctor and Nudinka by Becker et al. (1995) based on 16 different primer combinations. In contrast, RFLP markers, both from genomic clones and cDNA clones, showed higher polymorphism rates (27.1% and 15.3%, respectively; Heun et al. 1991), that were also observed between Igri and Franka (28%; Graner et al. 1991). In addition, 35.3% of RFLP clones showed polymorphisms between Morex and Steptoe when *Eco*RI was used as the restriction enzyme. Our datasets appear to suggest that AFLP markers also show lower polymorphism rates in barley. However, data are still too limited to allow one to generalise this conclusion to other species or populations.

Common markers among mapping populations

Due to the frequent exchange of RFLP probes among barley researchers, many common RFLP probes have been used as locus-specific markers and have been mapped on independent mapping populations. These

Table 3 AFLP polymorphism rates among 16 and between six pairs of barley lines

Primer combinations	16 Lines[a]		HT		SM		IF		PN		AP		LV		6 Crosses	
	TOT[b]	PR(%)[c]	TOT	PR(%)	TOT	PR(%)	TOT	PR(%)	TOT	PR(%)	TOT	PR(%)	TOT	PR(%)	TOT	PR(%)
E32M55	75	63	49	22	54	33	46	13	47	11	48	21	51	24	295	21
E32M61	79	46	58	12	65	20	65	20	59	5	59	9	67	33	373	17
E33M54	113	48	88	14	86	19	86	16	86	8	88	10	97	31	531	17
E33M55	89	49	63	5	69	17	67	15	63	13	64	17	71	21	397	15
E33M58	65	59	40	8	49	33	44	21	45	18	46	20	50	38	274	23
E33M61	106	67	72	29	76	38	67	22	68	13	71	23	78	39	432	28
E35M48	99	57	68	16	76	30	71	23	65	8	71	14	76	34	427	21
E35M54	84	43	68	9	68	16	71	14	66	5	71	17	72	22	416	14
E35M55	69	58	47	13	46	15	52	22	47	15	52	25	53	28	297	20
E35M61	92	59	68	18	73	23	68	21	70	11	74	24	72	29	425	21
E38M50	118	59	82	18	91	33	86	23	83	19	83	22	92	30	517	25
E38M51	95	47	72	7	76	18	70	10	70	9	71	10	75	25	434	13
E38M54	88	57	62	13	67	16	68	24	61	8	66	21	70	30	394	19
E38M55	64	56	48	17	49	25	44	18	47	13	50	20	49	33	287	21
E39M55	75	53	56	13	60	32	63	22	52	6	59	17	55	22	345	19
E39M61	98	59	61	8	71	24	61	16	64	20	66	23	70	33	393	21
E42M48	93	66	57	12	62	34	60	23	59	20	60	18	71	41	369	26
E42M51	91	53	62	13	60	20	64	16	64	16	60	13	68	29	378	18
E44M49	109	51	75	7	81	22	81	21	80	13	81	12	89	24	487	17
E44M54	118	47	94	9	100	26	91	10	92	8	94	15	98	21	569	15
E44M58	70	44	56	4	57	19	53	11	58	7	58	16	63	29	345	15
E45M49	122	59	88	14	97	33	86	20	86	15	83	17	96	29	536	22
E45M55	101	60	65	19	76	28	73	19	69	13	71	21	81	35	435	23
E45M58	75	64	54	20	49	25	46	24	45	20	52	29	51	18	297	23
Total	2188	55	553	13	1658	25	1583	19	1546	12	1598	18	1715	29	9653	20

[a] "16 lines" data from 16 barley lines; HT, SM, IF, PN, AP and LV represent the parent pairs of Harrington and TR306, Steptoe and Morex, Igri and Franka, Proctor and Nudinka, Apex and Prisma, and L94 and Vada, respectively; "6 Crosses" lists the accumulated data from these six parent pairs.
[b] Total number of bands
[c] Polymorphism rates

bridge markers enable the construction of integrated maps and comparison of independent maps (Langridge et al. 1995; Qi et al. 1996). The AFLP profiles generated with 16 barley lines in this paper could serve as standard references. Bands with only 1 bp difference migrate differently in gels and can be distinguished (Vos et al. 1995). Fragments with 16 (6 and 4 bases for *Eco*RI and *Mse*I sites, respectively, and 6 selective bp) identical base pairs and the same mobility in gels are most probably highly homologous and hence locus specific.

The present study indicates that common (assuming locus specificity) markers among populations can clearly be identified and may be used as bridge markers for the comparison of maps or assignment of linkage groups to chromosomes. By comparing the parent pair Steptoe and Morex with L94 and Vada, about 150 shared polymorphic AFLP markers were identified based on the 24 primer combinations listed in Table 3. Further survey indicated that 65 and 21 AFLP markers were common to three (Steptoe × Morex, Igri × Franka and L94 × Vada) and four parent pairs (Steptoe × Morex, Harrington × Tr306, Igri × Franka and L94 × Vada), respectively. Three clear AFLP markers, E38M55-618, E39M55-162 and E42M51-94, were common to five parent pairs (Steptoe × Morex, Harrington × Tr306, Igri × Franka, Proctor × Nudinka and L94 × Vada). These common markers are thus extremely useful for bridging maps. If the locus specificity of common markers is confirmed in future, AFLP markers will greatly contribute to merging marker and other genetic data into one integrated genetic map of barley.

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