

Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories

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Abstract

A number of PCR-based techniques can be used to detect polymorphisms in plants. For their wide-scale usage in germplasm characterisation and breeding it is important that these marker technologies can be exchanged between laboratories, which in turn requires that they can be standardised to yield reproducible results, so that direct collation and comparison of the data are possible. This article describes a network experiment involving several European laboratories, in which the reproducibility of three popular molecular marker techniques was examined: random-amplified fragment length polymorphism (RAPD), amplified fragment length polymorphism (AFLP) and sequence-tagged microsatellites (SSR). For each technique, an optimal system was chosen, which had been standardised and routinely used by one laboratory. This system (genetic screening package) was distributed to different participating laboratories in the network and the results obtained compared with those of the original sender. Different experiences were gained in this exchange experiment with the different techniques. RAPDs proved difficult to reproduce. For AFLPs, a single-band difference was observed in one track, whilst SSR alleles were amplified by all laboratories, but small differences in their sizing were obtained.

Abbreviations: RAPDs, random-amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; SSR, microsatellites or simple sequence repeats; GSP, genetic screening package

Introduction

Advances in DNA sequencing, data analysis and PCR have resulted in powerful techniques which can be used for the characterisation and evaluation of germplasm and genetic resources, and for the identifica-

tion of markers for use in breeding programmes. For the wide-scale application of these techniques, it is important that they are suitable for use in network activities in which many laboratories may be involved in coordinated actions and in which common data-bases are continually fed with data from all the participat-

ing groups. It is essential for such activities that the different screening techniques employed can be standardised to yield reproducible results across laboratories, so that direct collation and comparison of the data are possible. In the present study, an account is given of reproducibility testing of three popular molecular marker systems.

Random-amplified polymorphic DNA (RAPDs) involve the use of a single 'arbitrary' primer (purchasable from commercial companies) in a PCR reaction and result in the amplification of several discrete DNA products. Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands, that are complementary to the primer and sufficiently close together for the amplification to work. In RAPDs, the amplification products are separated on agarose gels in the presence of ethidium bromide and visualised under ultraviolet light [1, 2]. It is now widely recognised that to obtain reproducible band profiles on the gels it is absolutely essential to maintain consistent reaction conditions. Numerous studies have reported the separate effects of altering different parameters, ratio of template DNA primers, concentration of *Taq* polymerase and Mg concentration on the bands obtained [3–8]. A corollary of these experiments is that RAPD profiles should be reproducible among laboratories provided that all details of the reaction conditions are standardised and strictly adhered to.

Amplified fragment length polymorphism (AFLP) is another PCR-based method which first involves restriction digestion of the genomic DNA. Adapters are ligated to the ends of the restricted fragments and either a pre-selection step performed using magnetic beads followed by a round of selective PCR, or two selective rounds of PCR amplification are applied [9, 10]. The amplified products are separated on a sequencing gel and can be visualised using radioactive or fluorescent labelling. All the current evidence suggests that AFLPs are as reproducible as restriction fragment length polymorphism (RFLP). They should therefore be highly suited to network experiments.

Microsatellites or simple sequence repeats (SSRs) are highly mutable loci which may be present at many sites in a genome [11, 12]. As the flanking sequences at each of these sites may be unique, once SSR loci are cloned and sequenced, primers can be designed to the flanking sequences. The resultant sequence tagged microsatellite usually identifies a single locus which, because of the high mutation rate of SSRs, is often multi-allelic. Alleles which differ in many base pairs

of length can be resolved on agarose gels but often SSRs are visualised on sequencing gels where single repeat differences can be resolved and, thus, all possible alleles detected. SSRs provide highly informative markers because they are co-dominant (unlike RAPDs and AFLPs) and generally highly polymorphic [11–13]. The nature of the PCR-based assay used in their amplification and detection (i.e. the use of specifically designed primers based upon the flanking sequences) suggests that they should be highly reproducible between laboratories.

To test the standardisation and reproducibility of RAPD, AFLP and SSR markers, network experiments were undertaken by a number of European laboratories. For each technique, an optimal system was chosen, i.e. a system which had been standardised and used routinely by one of the participating groups, and used to construct a genetic screening package (GSP). The 'sender' laboratory distributed the GSP to different participating laboratories (recipients) in the network and the results obtained were compared with those of the original sender. Different experiences were gained in the network experiments for RAPDs, AFLPs and SSRs.

Materials and methods

The laboratories that took part in the reproducibility experiment are listed in Table 1. A GSP was prepared for each technique (RAPDs, AFLPs and SSRs) as follows.

RAPD Genetic Screening Package

The RAPD GSP was assembled by the Milan group (L2) and distributed to all of the other 8 laboratories. The RAPD GSP comprised the following: (1) genomic DNA samples from two clones of poplar (*Populus × euramericana*), Adige and I-214, extracted by the method described by Castiglione *et al.* [14]; (2) two decamers primers: –FS-25 5'-CTTGCCACG (Deca-12 [14]) and FS-27 5'-AGCCGGCCTT (Deca-10 [14]); (3) *Taq* polymerase (DynaZyme) and 10× PCR buffer (both Finnzymes Dy, Finland); (4) agarose (1% Metaphor/ 1% SeaKem LE, FMC BioProducts) and (5) a detailed protocol. Primers and genomic DNA were ethanol-precipitated and sent in 500 µl micro-fuge tubes to all participants. PCRs were performed as described in Castiglione *et al.* [14]). PCR reaction mixes consisted of 1× reaction buffer (1.5 mM MgCl₂,

Table 1. Participants of the reproducibility test network.

Lab group no.	Organisation
L1	IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Bristol, BS18 9AF UK
L2	Dipartimento di Biologia, Univeristà di Milano, 20133 Milano, Italy
L3	CPRO-DLO, PO Box 16, Wageningen 6700 AA, Netherlands
L4	Zeneca Seeds, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, UK
L5	Institut für Allgemeine Botanik, Ohnhorstrasse 18, 22609 Hamburg, Germany
L6	Università degli Studi di Firenze, Dipartimento di Biologia Animale e Genetica. 17, Via Romana, 50125 Firenze, Italy
L7	Dipartimento di Scienze Ambientali, Università degli studi di Parma, Via delle Scienze, 43100 Parma, Italy
L8	Laboratory of Gene Technology, Catholic University of Leuven, Willem de Croylaan 42, Leuven 3001, Belgium
L9	Departamento de Genetica, Facultad de Biologia, Universidad Complutense, Ciudad Universitaria, Madrid 28040, Spain

10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton x-100), dNTPs (200 μ M of each), 1 ng/ μ l primer, 0.5 ng/ μ l genomic DNA and 0.01 U/ μ l *Taq* polymerase. Only one DNA sample and one primer were added to any single reaction. All reaction volumes were 10 μ l (except for those of laboratory L1, where 25 μ l reaction volumes were found to be necessary), overlaid with a drop of mineral oil. A standard thermocycling programme was used in all laboratories:

1 cycle (an initial denaturing step) of 180 s at 92 °C
45 cycles of 45 sec at 92 °C, 60 s at 35 °C, 120 s at 72 °C

1 cycle (final extension) 10 min at 72 °C, then soak at 10 °C

Since different PCR machines were available in the different laboratories (MJ; Perkin Elmer Cetus 480; Hybaid Omnigene; Biometra; Techne), a thermoprofile was printed from the PCR machine used by L2 and copied to the recipient laboratories. Each recipient was asked to programme their PCR machines so that the thermoprofile obtained by L2 was copied. A standard ramp set was used by all groups.

Electrophoresis was performed in 4mm thick agarose gels (1% Metaphor/ 1% SeaKem LE, FMC BioProducts) with 1 \times TAE buffer (Stock solution 50 \times : 242 g of Tris base (Biotechnological grade) in 700 ml of deionised distilled water) for 3 h at 70 V, constant voltage. The gels were stained with 0.5 μ g/ml ethidium bromide, visualized on a 302 nm UV transilluminator and photographed with a Polaroid MP4, using Polaroid Type 667 film.

AFLP Genetic Screening Package

The AFLP GSP was prepared by the group at Zeneca (L4) and was distributed to labs L1, L2, L3, L5, L7 and L8. It contained the follow-

ing reagents: (1) genomic DNA from two sugar beet clones BOA113 and BOA714 S₁ 5127; (2) 'prepared DNA template from the same two sugar beet clones attached to Dynabeads; (3) adaptors *Pst*I 5'-bio-CTCGTAGACTGCGTACATGCA-3' / 3'-CATCTGACGCATGT-5' and *Mse*I 5'-GACGATGAGTCCTGAG-3' / 3'-TACTCAGGACTCAT-5'; (4) PCR primers *Mse*I.1 5'-GATGAGTCCTGAGTAAgaa, *Pst*I.1 5'-GACTGCGTACATGCAGac, *Pst*I.3 5'-GACYGCGTACATGCAGca, *Pst*I.3 5'-GACYGCGTACATGCA Gca. A number of reagents were listed as required for AFLP experiments, but were not contained in the package. These included (1) enzymes and reaction buffers (*Mse*I, *Pst*I, T4 kinase, *Taq* polymerase, ligase) (2) 'One Phor All' buffer, Pharmacia (3) ATP and dNTPs solutions, (4) ³³ γ P-dATP (Amersham); (5) Dynabeads M280 Streptavidin (Dyna) and (6) reagents for 4% denaturing polyacrylamide gel electrophoresis and autoradiography. The AFLP preparations and reactions, denaturing polyacrylamide gel electrophoresis and autoradiography were all carried out as described by Zabeau and Vos [9].

The two primer combinations were used against the Dynabead bound-templates prepared by the individual laboratories, as well as the controls supplied in the package and the resultant patterns compared. Finally, all Dynabead-bound templates were sent to L4 for AFLP analysis and the patterns compared directly on a single gel.

SSR Genetic Screening Package

The SSR GSP was prepared by the group at CPRO (L3). Recipients of this GSP were L1, L4, L6, L7, L8 and L9. In each case, recipients were asked to amplify the products, run them on a gel and size the alleles. The GSP contained: (1) genomic DNA from

two cultivars of tomato (*Lycopersicon esculentum*) cv. Vision and cv. Roma and two accessions of *L. peruvianum*, 372 and 1333; (2) PCR primers: LEGAST1 locus forward primer 361/reverse primer 362 [15] and LEMDDN locus forward primer 16166/reverse primer 16167 [15]; (3) a sequencing reaction as a size standard (four tubes containing each about 9 μ l of the different sequencing reactions of pGEM-3Zf(+)); (4) electrophoresis and silver staining instructions. Reagents required for the SSR experiments, but not contained in the GSP were: (1) *Taq* polymerase and reaction buffer (Gibco-BRL); (2) dNTPs solution; (3) electrophoresis and silver staining reagents.

PCR reaction mixes were 25 μ l of 1 \times reaction buffer (20 mM Tris-HCl pH 8.4, 50mM KCl, 1.5 mM MgCl₂, 0.05% W-1), dNTPs (100 μ M of each), 2 ng/ μ l of each primer, 0.4 ng/ μ l genomic DNA and 0.01 U/ μ l *Taq* polymerase, overlaid with a drop of light mineral oil. The thermocycling programme used for the Perkin Elmer-Cetus \ MJ thermocyclers was: one cycle (an initial denaturing step) at 94 °C for 5 min; 30 cycles at: 55 °C for 1 min (annealing), 72 °C for 2 min (extension) and 94 °C for 1 min (denaturing); one cycle at 55 °C for 1 min; one cycle (final extension) at 72 °C for 7 min, soak at 4 °C. The PCR amplification products were stored at -20 °C until used.

For the Hybaid Omni Gene thermocycler, the thermocycling programme used was: one cycle at 35 °C for 1 s; one cycle at 94 °C for 3 min, to completely denature the template; 30 cycles at: 55 °C for 45 s (annealing), 72 °C for 105 s (extension) and 94 °C for 45 s (denaturing); one cycle at 55 °C for 45 s; one cycle (final extension) at 72 °C for 7 min and keep at room temperature.

A number of laboratories were unable to obtain satisfactory results with silver-staining of the PCR products. The labs included either α (³⁵S)-dATP or α (³³P)-dATP in the PCR reactions. PCR products were diluted and mixed with a formamide/bromophenol blue loading buffer. Samples were denatured at 80 °C for 5 min and loaded onto sequencing gels (6% polyacrylamide, 8 M urea) and run at 60 W constant power for 2 h. Gels were then either stained or subjected to autoradiography.

The length of the alleles was determined by comparison with sequencing reaction runs on adjacent gel tracks. A microsatellite profile photograph was included in the GSP, against which the position of 153 bp was shown by an asterisk on the pGEM-3Zf(+) sequence (T lane) for use as a reference in length assessment. An exception was L9 who used their own

sequencing reactions (unpublished sequence, personal communication) for sizing the alleles.

Results

GSPs were exchanged among laboratories for the three different procedures, RAPDs, AFLP and SSRs. In the case of RAPDs, all recipient laboratories were able to perform the reactions without difficulty, but all had some previous experience with this technique. Few of the laboratories which received the AFLP GSP had prior experience of the procedure and many experienced difficulties when performing the reactions for the first time. In a few instances, the GSP had to be sent again. Eventually, all laboratories were able to carry out the procedure satisfactorily. Most of the recipient labs for the SSR GSP had no previous experience of sequence-tagged microsatellites. A major difficulty experienced was in obtaining satisfactory results with the silver-staining method and some laboratories were only able to complete the exercise through the use of autoradiography. Aside from this, all laboratories were able to carry out the SSR GSP reactions satisfactorily. In terms of the reproducibility of the results obtained, different experiences were gained with the different techniques and details are thus given separately for the different GSPs.

RAPDs

The RAPD profile obtained by the Milan group (L2) is given in Figure 1 and diagrammatically represented in Figure 2, along with representations of the band profiles obtained by the other 8 laboratories with primer FS-25 (Figure 2A) and primer FS-27 (Figure 2B). In Figure 2, the results from the two DNA samples of cultivars Adige and I-214 are shown combined. Any bands which were polymorphic between them are both represented as dotted lines.

The original profile obtained by L2 with primer FS-25 contained 10 bands of variable intensity which ranged in size from 300 to 1200 bp of which a single band was polymorphic between cvs. Adige and I-214 (320 or 300 bp) (Figs. 1 and 2A). The diagrammatic representation of the profile shown in Figure 2A includes one band, of the highest M_r (ca. 4000 bp in length) which is not apparent in Figure 1, but has been observed in other RAPD gels by the sender laboratory (Sala, personal communication).

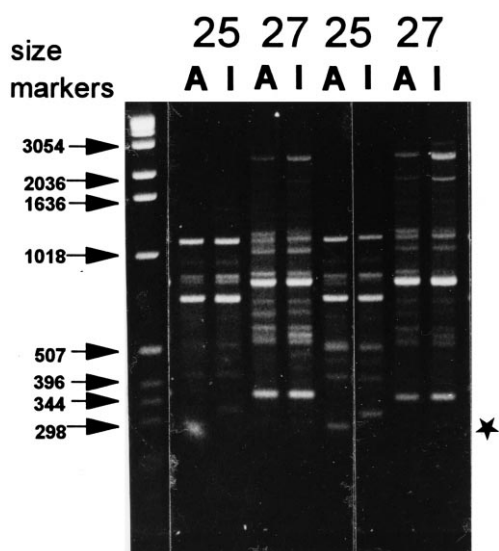


Figure 1. The RAPD GSP. The RAPD profile as obtained by the Milan group (L2). Size markers for assessing base pair lengths are shown in the ladder of the far left track. Two profiles using primers FS-25 (given as 25) and FS-27 (given as 27) are shown for the two cultivars Adige (A) and I-214 (I). The polymorphic bands observed with FS-25 are indicated by *.

The majority of recipient laboratories were able to amplify the same bands as the sender and to observe the same polymorphism but none reproduced the profile exactly. In general, all the bands of brightest intensity in the L2 profile were amplified by all eight other groups, but additional bands of comparable intensity were observed in profiles obtained by L8. The five bands within the size range of 1200 and 950 bp were amplified in 3 of the 8 other laboratories. The remaining groups (L1, L3, L7, L8 and L9) also amplified products within this range but occasional bands were absent, or present in addition, to those observed by L2. At least three of the four bands (one of which was polymorphic) produced below 550 bp were amplified in seven laboratories, but they were missing from the profile obtained by L6. All the recipient laboratories, with the exception of L4, amplified bands with a M_r greater than 1200 bp, which were not present in the majority of amplification profiles of the sender laboratory (L2). The band around 4000 bp in length, which appeared only occasionally in profiles of the sender, was observed by six out of eight recipients and appeared of brighter intensity in the profiles of L8 and L9.

In the RAPD profile obtained by L2, primer FS-27 gave the same 13 bands of 360–2500 bp for both cvs.

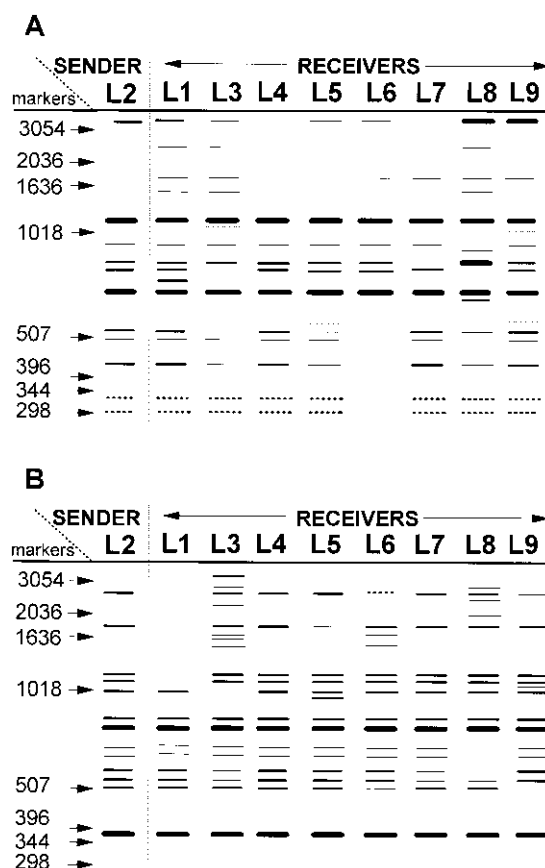


Figure 2. Diagrammatic representation of the RAPD profiles obtained by the sender, L2, and the 8 recipients, using the primers FS-25 (A) and FS-27 (B). Results for the two cultivars are shown combined and polymorphisms between them represented by dotted lines. The three different thicknesses of the bands in the diagram represent the relative intensities of bands in the profile, with the thickest band representing the brightest bands observed.

Adige and I-214, although some difference in intensity were present (Figs. 1 and 2B). All recipient laboratories reproduced the amplification of the two brightest bands (Figure 2B). Two labs (L7 and L4) reproduced the profile exactly, although some differences in intensity were present in the profile produced by L7. Many of the 10 bands within the size range 1200–500 bp were reproduced by all laboratories, but the profile of L1 was missing two bands, of L8 was missing three bands, and L3 one band, whereas those obtained by L5 and L9 had one band in addition. For the bands in the size range 1200 bp and above, the patterns obtained were more variable, with only two laboratories reproducing the profile exactly (L4 and L7). Four other laboratories could reproduce the bands of intermediate bright intensity, although intensities differed and one

group (L6) found a polymorphism here. Group L1 was entirely missing bands in this region, whilst L3, L6 and L8 had additional amplification products in this region.

AFLPs

The AFLP patterns obtained by the sender laboratory (L4) are shown in Figure 3A. The primer combination *Mse*I.1/*Pst*I.1 produced 64 bands, of which 8 were polymorphic between the sugar beet clones BOA113 and BOA714 S₁ 5127. The *Mse*I.1/*Pst*I.3 primer combination yielded 108 bands, of which 8 were polymorphic between the two sugar beet clones.

Initial problems were experienced by several groups in obtaining profiles in which a sufficient number of bands were amplified (up to 50% of bands were missing), but as these groups became more familiar with the procedure complete amplification was achieved.

The AFLP profiles obtained by the six recipient laboratories showed extremely high reproducibility as compared with the original profiles for the two primer combinations when either Dynabead-bound templates prepared by the individual laboratories from the DNA supplied in the GSP, or the Dynabead-bound template included as the GSP control, were used for the AFLP reactions. All AFLP profiles were identical with the exception of a single difference in the profile obtained by L3 using the *Mse*I.1/*Pst*I.1 primer combination on Dynabead-bound template DNA prepared by this group for the clone BOA714 S₁ 5127 (Figure 3B). To further clarify this discrepancy, the Dynabead-bound templates prepared by each group were sent to L4 for AFLP analysis so that the patterns obtained using templates from the recipient laboratories could be compared directly on a single gel by the sender, L4. The absence of the band in AFLPs of the Dynabead-bound DNA template obtained by L3 were confirmed in these later exchanges.

SSRs

The SSR GSP prepared by L3 comprised materials required for amplification of SSRs using two primer pairs for loci LEGAST1 and LEMDDN in two tomato cultivars and two *L. peruvianum* accessions [15]. Figure 4A shows the 13 amplified products as detected by group L3 using silver staining. The 11 alleles obtained differed in size between tomato and *L. peruvianum* for both SSR loci and between the two tomatoes and the two *L. peruvianum* accessions for the LEMDDN locus

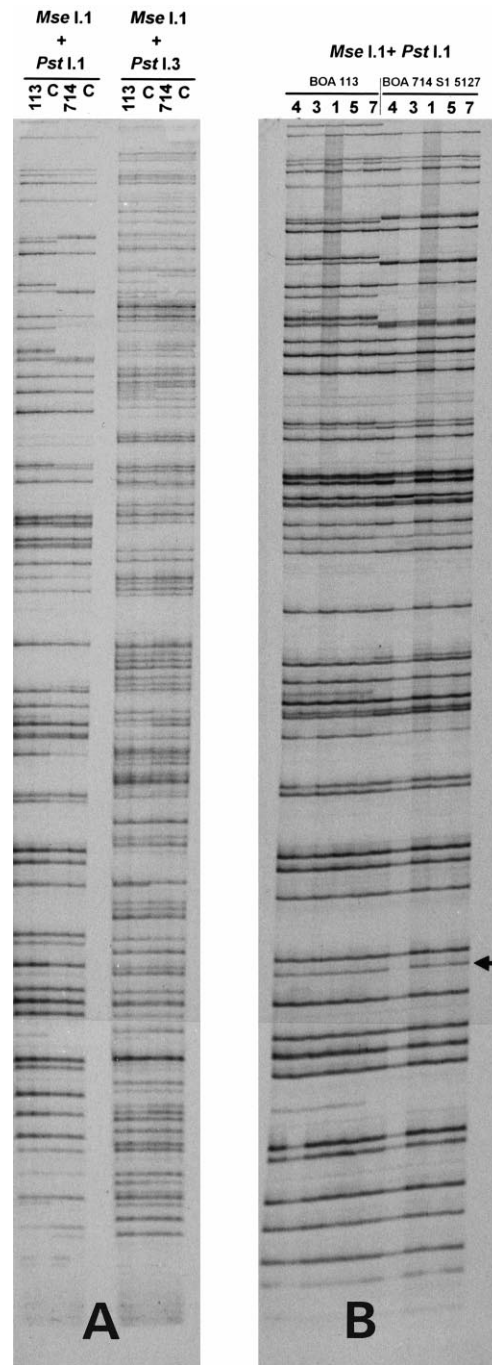


Figure 3. The AFLP GSP. A. Original AFLP profiles obtained by the sender (L4) for the two primer combinations *Mse*I.1 + *Pst*I.1 and *Mse*I.1 + *Pst*I.3 for the sugar beet clones BOA113 (or shown by 113) and BOA714 S₁ 5127 (or shown by 714). Profiles are given for DNA of the sample sent to all recipients (113 and 714) and for template-bound control DNA, C, also sent to recipients. B. AFLP profiles obtained by the sender L4 for the primer combination *Mse*I.1 + *Pst*I.1 using DNA-bound templates prepared by the recipient laboratories of the two sugar beet clones BOA113 and BOA714 S₁ 5127. The absence of a single band in the track of L3 is marked by the arrow.

(Figure 4A and Table 2). For the LEGAST1 locus, the alleles differed in size between the two *L. peruvianum* accessions but were identical for the two cultivars of tomato (Fig 4A and Table 2). All the alleles at the LEGAST1 locus, in all four samples, showed stutter bands, whilst less stuttering occurred with alleles of LEMDDN, particularly in *L. peruvianum*.

All the recipients of this GSP (L1, L4, L6, L7, L8 and L9) reproduced the amplification of the SSR alleles observed in the original GSP. No alleles were missing or gained. All groups found more stuttering for the LEGAST1 locus but the appearance of the alleles in relation to the number of stutter bands differed between groups. The least stutter was observed with ³⁵S labelling and the greatest with ³³P, by group L9, where exposure was allowed to proceed for longer than in the case of L1, where ³³P was also used.

Two of the five recipient laboratories (L1 and L6) obtained exactly the same size of alleles at both loci in both the tomato cultivars and the *L. peruvianum* accessions. The three remaining groups also obtained the same sizes for some of the alleles, particularly for LEGAST1 locus in tomato cvs. Vision and Roma (L4, L7), but obtained small differences in sizing the other alleles (Table 2). L6, which used the silver staining method for detection, sized the alleles identically to those of L3. L7, which also used silver staining, obtained similar sizes for 9 of the 13 amplification products, but 4 alleles differed by 1 bp compared with L3. The main differences in allele sizes were observed by L9, but considerable stuttering was also experienced by this group using ³³P and this group also used a different sequencing reaction to size the alleles.

Discussion

A number of reproducibility experiments have been reported in the literature, in which the robustness of DNA marker systems have been tested. In the majority of instances, this has involved reproducibility tests within a laboratory, although experiments testing the ability of different laboratories to achieve identical RAPD profiles have also been described [16]. In the present article, the reproducibility of three marker systems (RAPDs, AFLPs and SSRs) have been compared in network experiments involving several European laboratories. Different experiences were gained with RAPDs, AFLPs and SSRs.

RAPDs were found to be easy to perform by all groups, but reproducibility was not achieved to a sat-

isfactory level. Systematic tests on the effects of some factors influencing RAPD patterns have been carried out by several research groups [e.g. 3, 4, 7, 8] and there are many reports on the importance of *Taq* polymerases and thermocycling machines in the optimisation of PCR reactions conditions for RAPDs [e.g. 5, 6, 7]. Variability in RAPD profiles due to the use of different PCR machines and polymerase has also been experienced by the present authors. In a previous network reproducibility experiment (unpublished results) each laboratory prepared their own package according to their favoured method of performing RAPDs and on species with which they had prior experience. RAPD GSPs were then exchanged and each laboratory attempted to reproduce the results of the others. A very high rate of variability was found in this experiment, with only 2 profiles out of the 9 × 9 exchanges reproduced. In the experiment described here, a single GSP, containing the *Taq* polymerase, buffer etc., was distributed by one laboratory and thermoprofiles stipulated in order to reduce the effect of using different PCR machines. Despite this, variation in band profiles were observed for both primers used and even for bands of strong intensity. This supports earlier findings of irreproducibility in RAPD profiles performed by a number of laboratories [16]. Skroch and Nienhaus [16] have further examined the impact of this irreproducibility on the scoring of RAPDs. When expressed as the percentage of RAPD bands scored that were also scored in replicate data, only 75% reproducibility was obtained for 50 RAPD primers.

AFLPs were found to be initially difficult to perform by most groups, whose first attempts produced profiles in which over 50% of bands were missing. With greater familiarity, this problem resolved and the AFLPs profiles obtained subsequently showed extremely high reproducibility. This confirms previous reports on the reproducibility of AFLP profiles [18–20]. In the experiment described here, only a single difference was observed (the absence of a band) in the AFLP profile obtained with one primer combination (*Mse*I.1/*Pst*I.1), in one of the two clones, using Dynabead-bound template DNA prepared by one of the recipient laboratories (L3). When the AFLP patterns obtained using templates from all recipients were compared on a single gel by the sender laboratory, the absence of the band was confirmed. This contrasts with the experience of Janssen *et al.* [19] who prepared templates from total DNA of six *Aeromonas* strains, extracted on 6 separate occasions and found no differences in the AFLP obtained. However, the level of difference

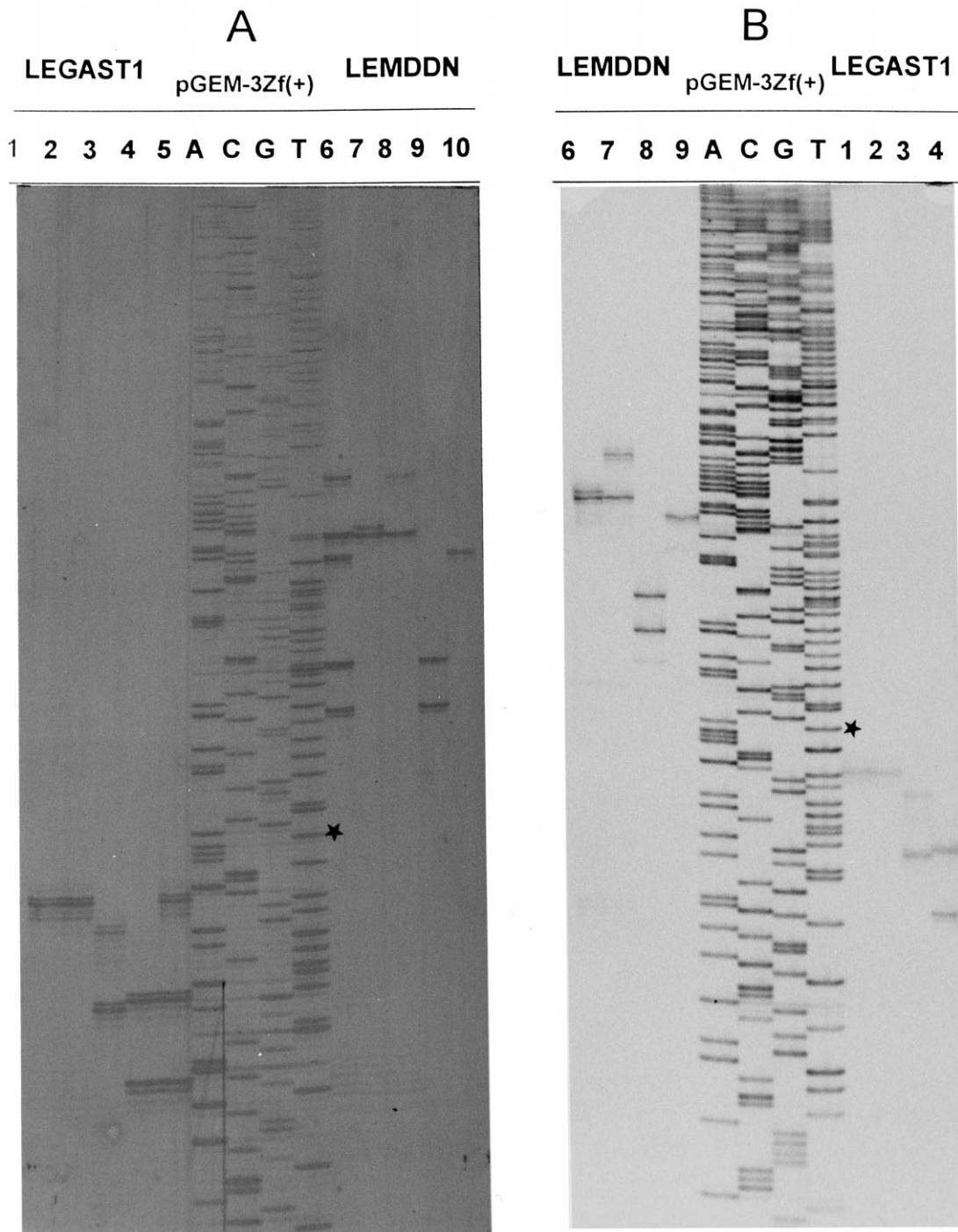


Figure 4. The SSR GSP. A. Original silver-stained profile of the SSR alleles for the two loci LEGAST1 and LEMDDN; lanes 1-5, LEGAST1 locus; lane 1, *Lycopersicon esculentum* cv. Vision; lane 2, *L. esculentum* cv. Roma; lane 3, *L. peruvianum* 372; lane 4, *L. peruvianum* 1333; lane 5, mixture of PCR reactions for *L. esculentum* cv. Vision and *L. peruvianum* 1333; lanes A, C, G and T, sequencing lanes for the pGEM-3Zf(+) vector; lanes 6-10, LEMDDN locus; lane 6, mixture of PCR reactions for *L. esculentum* cv. Roma and *L. peruvianum* 1333; lane 7, *L. esculentum* cv. Vision; lane 8, *L. esculentum* cv. Roma; lane 9, *L. peruvianum* 372; lane 10, *L. peruvianum* 1333. * indicates base pair number 153 of the pGEM-3Zf(+) sequence. B. Example of a recipient SSR result (L4), in this case, detected using autoradiography. Note that the labelling of the tracks is the same as for (A) but the LEMDDN and LEGAST1 are in the inverse order. * indicates base pair number 153 of the pGEM-3Zf(+) sequence.

Table 2. Estimated SSR allele sizes for the two loci LEGAST1 and LEMDDN in the two tomato cultivars Vision and Roma and the two clones of *L. peruvianum* derived by different recipients of the SSR GSP. The sender laboratory was L3. Allele sizes which differed from those of the sender are given in bold. The method used for detection is shown in the right column.

Lab	LEGAST1				LEMDDN				Detection
	Vision	Roma	372	133	Vision	Roma	372	133	
L3	145	145	141/ 131	132/ 123	212/ 211	229/ 211	182/ 173	206	silver
L1	145	145	141/ 131	132/ 123	212/ 211	229/ 211	182/ 173	206	³³ P
L4	145	145	141/ 131	132/ 122	212/ 211	229/ 212	182/ 173	206	³⁵ S
L6	145	145	141/ 131	132/ 123	212/ 211	229/ 211	182/ 173	206	silver
L7	145	145	141/ 130	131 / 122	212/ 211	229/ 211	181 / 173	206	silver
L9	143	143	139 / 128	130 / 121	211 / 210	222 / 210	182/ 174	206	³³ P

observed here is similar to that reported by Becker *et al.* who used DNA prepared from two barley cultivars in 3 independent AFLPs on two different gels and observed that ‘almost all the bands visible in the gel’ could be reproduced consistently [20]. Furthermore, Huys *et al.* [21] found correlations of between 95.0 and 98.5% for the reference samples, when AFLP reproducibility in *Aeromonas* was assessed using a single strain as an internal reference during each electrophoresis run. Since AFLPs are based upon restriction digestion of DNA, the quality of the extracted DNA and the method of extraction could affect the profiles obtained. However, in the present study, all the DNA was extracted by the sender and the differences observed were introduced at the template preparation step. Although AFLPs are insensitive to template concentration, differences may occur when templates are excessively diluted [10]. This was not the case here, however, and the absence of the band can only be placed down to experimental errors incurred in performing the AFLP procedure [21].

In the case of the SSR reproducibility experiment, some recipients experienced difficulties with the silver-staining procedure but the alleles of the original GSP were reproduced by all groups. These results are in general keeping with previous claims on the robustness of microsatellites [13, 22–24]. Small differences (in the order of one base pair) were obtained in the size estimation of the alleles and were also observed in the amount of stutter bands. Recipient laboratory L9

obtained allele sizes differing by several base pairs from the original, but this was attributed to the fact that a different sequence was used as the standard. The finding of one bp difference is within the range of scoring errors previously reported for SSRs [25]. In the present study, the accuracy of size estimation was found to be influenced by: (1) the amount of stutter; (2) the choice of band selected as the allele and; (3) the distance of the allele from the sequence used as the standard; factors (1) and (2) were related. The inclusion of a sample containing mixtures of all the alleles in each gel may improve accuracy of sizing the same alleles in different samples. Differences in the sizes of alleles did not appear to be consistently affected by the use of silver staining compared with autoradiography. This contrasts with earlier results by Bowers *et al.* [25], where allele sizes for 3 out of 4 loci were found to be consistently larger by 1 bp, and for the fourth locus by 2 bp, with silver staining compared with automated fluorescent detection. The size off-set was consistent for all cultivars and all alleles and was deemed to be within the error typical of allele size estimates based on different methods [26].

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