Theor Appl Genet (1999) 98: 107–112

A. Prevost · M. J. Wilkinson

A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars

Received: 7 May 1998 / Accepted: 15 July 1998

Abstract Commercial scale fingerprinting of potato cultivars is made difficult by the need for speed, reliability and the ability to distinguish between large numbers of genotypes. There are also problems in extrapolating the results of small experimental studies to predict the performance of techniques or primers for larger applications. The potential of ISSR-PCR for fingerprinting purposes was evaluated using four primers on 34 potato cultivars. The complex band profiles generated were reproducible between repeat PCRs, DNA extractions, electrophoreses and gel scorings. Two primers were each able to distinguish all cultivars. The combined use of any two of the four primers also allowed complete diagnosis. It is concluded that ISSR-PCR provides a quick, reliable and highly informative system for DNA fingerprinting that is amenable for routine applications. Two possible correlates of the ability of primers to distinguish between genotypes were then examined. Marker Index failed to correlate significantly with genotype diagnosis, but a strong and seemingly linear relationship was observed between Resolving Power of a primer and its ability to distinguish genotypes ($r^2 = 0.98$). Resolving Power of one or a pair of primers was found to provide a moderately accurate estimate of the number of genotypes identified. Possible implications for future studies on DNA fingerprinting are discussed.

Key words DNA fingerprinting · ISSR-PCR · *Solanum tuberosum* · Resolving Power

Communicated by J. W. Snape

A. Prevost · M. J. Wilkinson (✉)
Department of Agricultural Botany, School of Plant Sciences, The University of Reading, Whiteknights, P.O. Box 221, Reading, RG6 6AS, UK

Introduction

The potato seed industry and associated statutory bodies require a reliable means of cultivar identification that can be applied routinely to large numbers of samples. This task is made difficult by the fact that there are over 1000 potato cultivars world-wide (Chase 1989), and significant numbers of new cultivars are being registered annually. Several molecular approaches have been evaluated for cultivar diagnosis, including restriction fragment length polymorphism (RFLP, Gebhardt et al. 1989), random amplified polymorphic DNA (RAPD, Demeke 1993) and simple sequence repeats-polymerase chain reaction (SSR-PCR, Provan et al. 1996; Kawchuk et al. 1996). Inter-simple sequence repeat-PCR (ISSR-PCR) has yet to be applied for potato cultivar identification but has been used to generate DNA fingerprints of maize (Kantety et al. 1995), wheat (Nagaoka and Ogihara 1997) and oilseed rape (Charters et al. 1996) cultivars. Allainguillaume et al. (1997) also used ISSR-PCR to detect gene flow from *Solanum phureja* into potato dihaploids.

For practical reasons, most studies assessing new methods of cultivar diagnosis have utilised relatively modest collections of plants, although the number and composition of genotypes used varied widely. For instance, Hosaka et al. (1994) used 31 RAPD primers to distinguish 67 of 73 cultivars/breeding lines. In contrast, Sosinski and Douches (1996) screened 16 RAPD primers and used the 10 most informative to distinguish between all of 46 North American cultivars. Such variation complicates comparisons between studies. An alternative strategy would be to use a function that is strongly correlated to the proportion of genotypes identified but is independent of the number of genotypes studied. Here, we investigate two possible measures of the ability of primers or techniques to distinguish between genotypes (Marker Index and Resolving Power).

Marker Index (MI) was described by Powell et al. (1996) and used by Milbourne et al. (1997) to compare RAPD, amplified fragment length polymorphism (AFLP) and SSR-PCR analyses in genetic relationship studies. It was not evaluated as a measure of the ability of a primer or technique to distinguish between individuals. The alternative function, Resolving Power (Rp) is explained below.

In the study described here, ISSR-PCR was used to fingerprint a selection of potato cultivars. We then examined the relationship between two functions (MI and Rp, see below) and the ability of ISSR primers to distinguish between potato cultivars. The function showing the greatest correlation with genotype diagnosis was used to compare the value of ISSR primers for the identification of potato cultivars.

Materials and methods

Plant material

The 34 potato cultivars used in the study were received as tubers from the breeder's germplasm collection at the Scottish Crop Research Institute (SCRI), Dundee. *Solanum tuberosum* ssp. *andigena* (ADG 1683) obtained from the Commonwealth Potato Collection (also housed at the SCRI) was used as an outgroup.

Primers

Four primers (827, 834, 841 and 857) were selected in the current study. These were obtained from the University of British Columbia (set # 9), Vancouver, Canada and are listed below:

827: $[AC]_8G$ 834: $[AG]_8YT$ 841: $[GA]_8 \cdot YC$ 857: $[AC]_8YG$

(where Y = C or T).

PCR amplifications

DNA was extracted from 0.8 g of fresh young leaves according to the procedure of Doyle and Doyle (1987).

PCR reaction mixtures (20 µl) contained the following components/concentrations: 1 U of *Taq* DNA polymerase (Boehringer Mannheim), 1.5 m*M* $MgCl_2$ buffer (supplied with the enzyme), 0.3 µ*M* of primer, 0.2 m*M* of each dNTP (all Boehringer Mannheim) and 20 ng of template genomic DNA. The mixture was overlaid with mineral oil and subjected to PCR on a Hybaid Omnigene Thermocycler using the following programme: 30 cycles of 1 min at 94°C, 2 min at 55°C, 30 s at 72°C, with a final extension of 5 min at 72°C.

Electrophoresis of PCR products

Precast polyacrylamide gels (Cleangel 48S, Amersham Pharmacia Biotech) were rehydrated for 1 h in 112 m*M* TRIS acetate (pH 6.4). Loading buffer (2 µl, comprising 0.02% w/v bromophenol blue, 5 *M* urea) was added to the PCR amplification products (5 µl). The samples were then subjected to electrophoresis at 10°C on the Multiphor II flatbed system (Amersham Pharmacia Biotech) using the following three-stage programme: (1) 20 min at 200 V max, 20 mA max, 10 W max; (2) 50 min at 380 V max, 30 mA max, 20 W max; (3) > 30 min at 450 V max, 30 mA max, 20 W max. Electrophoresis was deemed to be complete when the blue dye front ran to the gel margin.

Staining

Bands were visualised by silver nitrate staining using the method of Bassam et al. (1991), with modifications as described by Charters et al. (1996). Great care was taken to ensure uniformity of band intensity between gels by stopping the developing reaction with reference to selected 'standard' bands within the profile.

Compilation of band profiles from hypothetical primers

PCR band profiles were produced for 34 cultivars using the four selected ISSR primers. Two independent PCRs were carried out for each primer, and the products were fractionated on separate gels. The band profiles of each gel were scored visually on two occasions using an illuminated light box. Consensus profiles were recorded on the basis of the presence (1) or absence (0) of bands at 75 band positions and assembled onto a data matrix. Band profiles of hypothetical primers were then compiled by randomly selecting 8–10 band positions from the 68 informative bands contained in the matrix. A total of 371 hypothetical primer profiles were generated in this way. The presence or absence of bands at each position was then scored for each of the 34 cultivars. The synthetic band profiles of each cultivar were then subjected to pairwise comparisons and UPGMA cluster analysis using the PAUP version 4.0 software package (Apple MacIntosh 1994) to determine the number of cultivars that could be distinguished.

Calculation of Marker Index (MI)

Marker Index was calculated as the product of two functions: DI (Diversity index) and EMR (Effective Multiplex Ratio). DI of a primer is defined as $1 - \Sigma p_i^2$, where p_i is the frequency of the ith allele (band). EMR of a primer is defined as "the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay" (Milbourne et al. 1997).

Calculation of Resolving Power

Resolving Power is based on the distribution of alleles within the sampled genotypes. The most efficient means of separating any group of taxa for identification purposes is to progressively divide the group into equal subgroups. Under optimal conditions, a collection of 64 individuals would separate into two subgroups of 32, then further subdivide into progressively smaller groups of 16, 8, 4, 2 and then 1. This provides the minimum path length for identification (in this case, six divisions) irrespective of the genetic makeup of the group being analysed. For most molecular fingerprinting systems, the division of genotypes into two groups is based on the presence or absence of a band at a particular position. Ideally, each band position would therefore be present in half of the genotypes and absent from the other half. Under optimal conditions for diagnosis, the use of a second band position would divide the taxa into quarters. Subsequent bands would subdivide further into roughly equal groupings. The perfect system would therefore contain large numbers of bands that would each divide the taxa into roughly equal halves. The ability of any two such band positions to subdivide a group into quarters would be dependant upon the absence of linkage and on the complementarity of the groups formed by each band individually. It is possible, for instance, that two band

positions would divide the taxa into identical halves. In practice, provided the taxa form a homogeneous set (i.e. are not comprised of distinctive subgroups such as cultivars of two species), then the more band positions considered, the greater the probability that two of them will divide the genotypes into complementary halves. It follows that the value of a primer/technique will be a function of how many band positions are generated and of how close to the optimal condition (division of taxa into equal halves) each band position is.

The value of a particular band position can be measured most simply then by its similarity to the optimal condition (50% of genotypes containing the band). This 'band informativeness' (I_b) can be represented into a 0-1 scale by the simple formula:

$I_b = 1 - (2 \times |0.5 - p|)$, where p is the proportion of the 34 genotypes containing the band.

The I_b value was calculated for all 68 informative ISSR bands that were scored in the study.

If all bands were optimally informative, then the most useful primer or technique would be simply those that generated the most band positions. Given that bands can be weighted according to their similarity to optimal informativeness, the ability of a primer or technique to distinguish between large numbers of genotypes could be represented by the sum of these adjusted values. This can be described as the Resolving Power of the primer (Rp), where:

$Rp = \Sigma I_b$.

The Resolving Power of 371 hypothetical primers and four ISSR primers was determined in this way.

Band profile reproducibility

Four replicate DNA extractions from leaves of cv 'Pentland Dell' and cv 'Brodick' were used to assess the consistency of band profiles using primer 834. The band profiles of 34 cultivars were subjected to standard electrophoresis conditions and scored visually on two occasions using an illuminated light box. Consistency of band profiles between replicate PCRs was assessed on triplicate ISSR PCRs on cv 'Morene' and cv 'Nicola' using primer 834.

Results

Cultivar identification

All four primers generated complex band profiles. These were scored by eye on two independent occasions and compiled into a data matrix. Band scores did not differ between repeat assessments or between gels for any of the primers used. The majority of band positions (68 out of 75 scored) varied between cultivars. These ranged from bands that were unique to 1 cultivar or absent only in 1 cultivar (poorly informative, 3 bands) to those which were present in approximately 50% of the cultivars (highly informative). A total of 17 bands (25%) were present in 14–20 cultivars (40–60%), and these were considered highly informative for diagnostic purposes.

There was only a poor association between the number of informative bands generated by primers and the proportion of the 34 genotypes that could be separated. The two primers that produced the most informative band profiles (primer 841, Fig. 1 and primer 834) were both able to distinguish all of the cultivars. On the other hand, profiles generated by primer 827 contained 10 variable band positions and could distinguish 30/34 cultivars, whereas primer 857 generated 11 variable band positions but could only identify 22/34 cultivars.

Band profile reproducibility

Four replicate DNA extractions were made from the leaves of cv 'Pentland Dell' and cv 'Brodick' and used to assess the consistency of band profiles generated using primer 834. No variation was observed in the position or presence of bands that had been selected for scoring. Similarly, triplicate ISSR-PCRs performed on

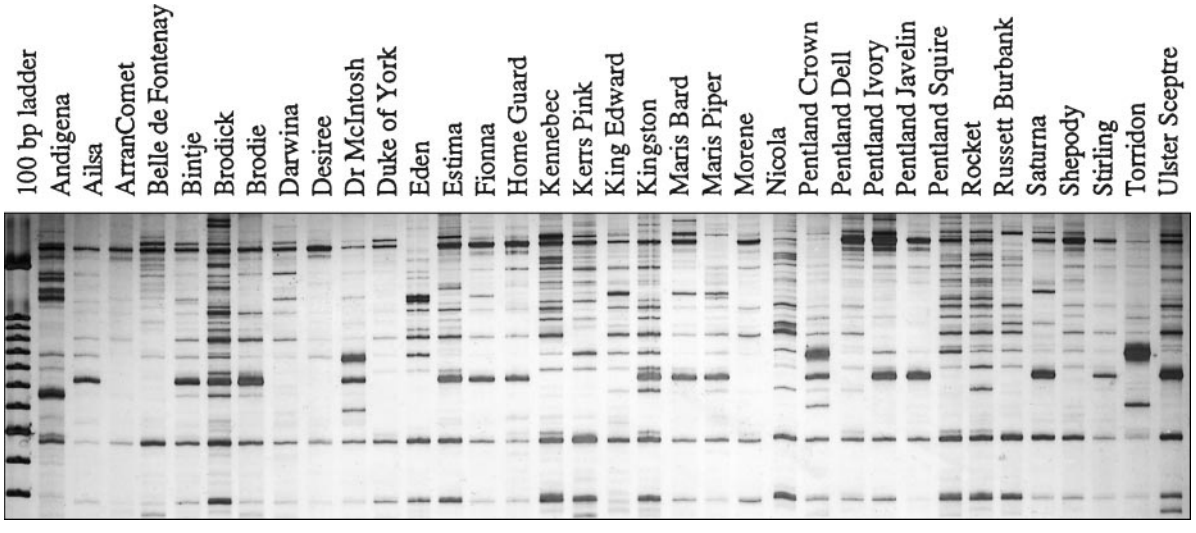

Fig. 1 Band profiles of 34 potato cultivars generated by primer 841

DNA from cv 'Morene' and cv 'Nicola' using primer 834 revealed no variation in band presence or position but did reveal subtle changes in relative band intensity. No differences were observed between independent scorings of any gel for any of the selected band positions.

Relationship between MI, Rp and cultivar identification

A total of 33 randomly selected hypothetical primers were used to compare the relationships between MI or Rp and the number of genotypes identified. There was no significant relationship between MI and the number of genotypes identified ($r^2 = 0.18$). There was a seemingly linear relationship, however, between Rp and the proportion of cultivars identified ($r^2 = 0.65$).

The nature of the relationship between Rp and genotype diagnosis was examined further using a total of 371 hypothetical primers producing 8, 10 or 12 band positions. A strong linear relationship ($r^2 = 0.98$) was observed between the Resolving Power of hypothetical primers and the proportion of the 34 cultivars that each was able to distinguish (Fig. 2). This relationship was unaffected by the number of band positions included in the hypothetical profiles, with those containing 8 band positions aligning in the same way as those with 10 or 12 band positions. The relationship was described by the equation $0.15x + 1.78 = Rp$, where x is the number of genotypes identified.

The strong relationship meant it was possible to estimate the number of genotypes that could be identified simply by calculating the Resolving Power of a hypothetical primer. It was noted, however, that the relationship appeared slightly less reliable at the upper limit of the graph. On average, primers with a Rp value of 6.9 were predicted to identify all 34 cultivars. Nevertheless, there were several primers with Rp values above this figure that were unable to distinguish all genotypes and others, with an Rp of < 6.9 that could separate all cultivars.

The relationship between cultivar identification and Rp was even maintained when band profiles generated by two hypothetical primers were combined. The collective Resolving Power of both primers fit to the same line as that described for individual primers (Fig. 3). It follows that it was possible to predict the approximate number of genotypes that could be identified by the combined use of two primers simply by adding their Resolving Power values.

Resolving Power of ISSR primers on potato genotypes

The Resolving Power of the four ISSR primers ranged from 4.6 for primer 857 to 12.5 for primer 841. Two of the ISSR primers (834 and 841) possessed high Rp values (10.6 and 12.5, respectively) and were able to distinguish all 34 cultivars. The remaining two primers (827 and 857) were both unable to fully separate all genotypes. Resolving Power provided a modest indication of the ability of these primers to distinguish between cultivars. The Resolving Power of primer 827 (7.7) suggested that 34 genotypes would be distinguished rather than the 30 actually separated. The Rp of primer 857 (4.6) inferred that 19 genotypes would be identified, whereas 22 were actually diagnosed.

Discussion

Any system to be used for the comprehensive fingerprinting of potato cultivars should be reproducible and able to distinguish between large numbers of closely related genotypes. Band profiles generated by ISSR-PCR in the present study were unaffected by replicated PCRs or DNA extractions. Furthermore, independent scorings of gels produced identical data sets. The protocol therefore appears to be sufficiently reproducible for

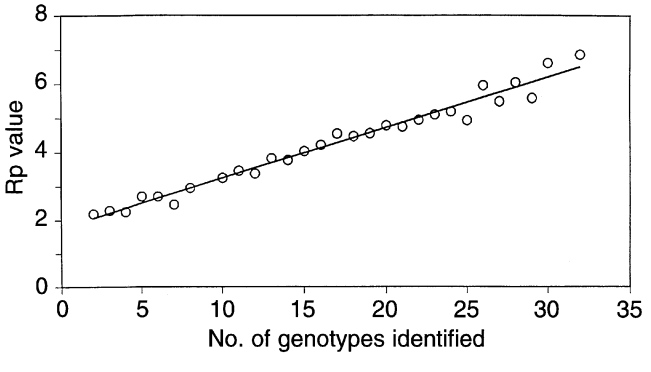

Fig. 2 Relationship between Rp and the number of genotypes distinguished using 371 hypothetical primers. Each point represents the mean Rp of 10 primers identifying the same number of genotypes. The regression line is described by the equation: $Rp + 0.15x + 1.78$, where x is the number of genotypes identified ($r^2 = 0.98$)

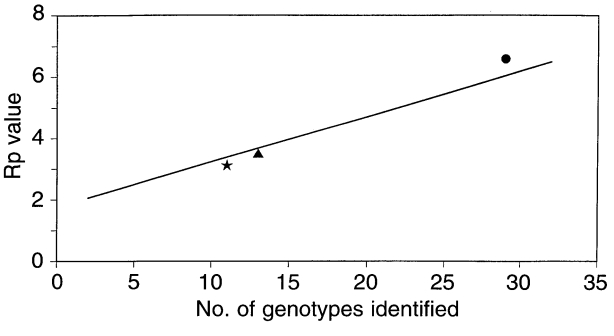

Fig. 3 Relationship between Rp and the number of genotypes distinguished using hypothetical primers A (★), B (▲) and the combination of both primers, A + B (●)

fingerprinting purposes. This is in concordance with previous studies where the reliability of the protocol was demonstrated between PCRs, DNA extractions and even laboratories when applied to cocoa (Charters et al. 1998) and oilseed rape (Charters et al. 1996).

Each of the four ISSR-PCR primers used here generated large numbers of polymorphisms. This was sufficient to allow all 34 genotypes to be distinguished using either of the two most informative primers (834 and 841). The combined use of any two of the four primers also allowed diagnosis of all cultivars. Thus, ISSR-PCR appears to produce reliable and highly polymorphic band profiles. This concurs with the work of Salimath et al. (1995) on the genus *Eleusine* in which ISSR analysis was found to generate more polymorphisms than either RFLP or RAPD analyses. A key additional feature of ISSR-PCR lies in its technical simplicity and speed. PCR products can be generated, fractionated and detected within 9 h. The technique seems to have potential, therefore, for the large-scale and systematic fingerprinting of potato clones.

Comparison of the diagnostic capacity of primers or techniques is almost invariably based on results from small-scale studies. Difficulties arise when attempting to make comparisons between such investigations or in predicting the performance of primers or techniques on a much larger scale. Milbourne et al. (1997) used Marker Index (MI) as a basis for comparing techniques. The function has not been evaluated as a measure of the ability of primers or techniques to diagnose genotypes. Results obtained in the present study suggest that there is little or no correlation between MI and the ability of primers to distinguish between genotypes. It follows that the practical value of this function for this purpose is limited and that an alternative correlate should be sought.

Resolving Power has been found to correlate strongly with genotype diagnosis and so has potential for a number of applications. It is possible that several primers included in a preliminary study are able to distinguish between all of the genotypes used. Such primers are the most likely to be selected for larger applications although there is currently no basis for comparing between them. In the present work, for example, two ISSR-PCR primers were each able to recognise all 34 cultivars used and so could not be segregated on the basis of their ability to diagnose genotypes. Nevertheless, they could be ranked according to their Rp values under the reasonable premise that primers with higher Rp values have a greater capacity to separate potato genotypes.

Resolving Power may also have value for comparisons between studies. It is often extremely difficult to compare the value of primers used in different investigations. For instance, it would be problematic to rank the performance of 31 primers able to distinguish 67 of 73 potato cultivars (Hosaka et al. 1994) against 10 that could separate 46 of 46 cultivars (Sosinski and Douches 1996). The use of Resolving Power might enable direct comparison of primers both within and between such surveys, provided the genetic make-up of the material used in each was roughly comparable.

Perhaps the most intriguing possibility, however, would be the prospect of being able to use the Rp value to predict the number of genotypes that could be resolved by one or more primers. In the present work, Rp value provided a moderately accurate guide to the numbers of genotypes that hypothetical and actual ISSR primers were able to differentiate. Furthermore, it was also possible to predict the discriminatory capacity of pairs of primers when used together from their combined Rp values. At present, it is only possible to speculate on whether the observed relationship extends beyond the 34 genotypes included in this study. There could be important implications, however, should the relationship hold true for expanded samples of genotypes. For example, the combined Rp value for the four ISSR-PCR primers used here was 35.4. This figure would suggest that, on average, their combined use should allow all genotypes to be distinguished up to a sample size of 224 genotypes. This assumes that the expanded set of individuals would contain similar levels of genetic diversity to those used here and would not include sports or mutant off-types. This possibility warrants further examination. It is important to stress, however, that Rp provides no information on the ability of a primer to reflect the genetic or taxonomic relationships of a group of genotypes under study. It does not, therefore, provide a measure of the overall value of a primer, particularly in studies of genetic distance.

The present work has shown that ISSR-PCR analysis is quick, reproducible and generates sufficient polymorphisms to have potential for large-scale DNA fingerprinting purposes. Resolving Power (Rp) of a primer has been found to correlate strongly with its ability to distinguish between genotypes. The function is well suited for comparing primers, primer-enzyme combinations or probe-enzyme combinations generated by RAPD, ISSR-PCR, AFLP or RFLP analyses. Crucially, Rp provides quantitative data allowing comparisons between primers (or probe-enzymes etc.), including those that are able to distinguish all genotypes examined in a study. It can also be used to predict the performance of groups of primers. Further work is required, however, to establish whether Rp determined in preliminary studies can accurately predict the performance of primers in larger scale fingerprinting experiments.

Acknowledgements We would like to express our gratitude to Dr. Stefan Houghton for helping to compile the figures and to Prof. Peter Caligari for his helpful advice. Thanks also to the European Union (FAIR programme) for providing financial support for this work.

References

Allainguillaume J, Wilkinson MJ, Clulow SA, Barr SNR (1997) Evidence that genes from the male parent may influence the morphology of potato dihaploids. Theor Appl Genet 94: 241–248

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991) Fast and sensitive silverstaining of DNA in polyacrylamide gels. Ann Biochem 196: 80–83

Charters YM, Robertson A, Wilkinson MJ, Ramsay G (1996) PCR analysis of oilseed rape cultivers using 5′ anchored SSR primers. Theor Appl Genet 92: 442–447

Charters YM, Culham A, End M, Hadley P, Wilkinson MJ (1998) The potential of anchored microsatellite analysis for cocoa germplasm characterization. In: Proc 12th Int Cocoa Res Conf., Salvador-Bahia, Brazil (in press)

Chase RW (1989) North American Potato Variety Inventory. Certification Section Potato Association of America, Orono, Me., USA

Demeke T, Kawchuk LM, Lynch DR (1993) Identification of potato cultivars and clonal variants by Random Amplified Polymorphic DNA analysis. Am Pot J 70: 561–569

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11–15

Gebhardt C, Blomendahl C, Schachtschabel U, Debener T, Salamini F, Ritter E (1989) Identification of 2n breeding lines and 4n varieties of potato (*Solanum tuberosum* ssp. *tuberosum*) with RFLP fingerprinting. Theor Appl Genet 78: 16–22

Hosaka K, Mori M, Ogawa K (1994) Genetic relationships of Japanese potato cultivars assessed by RAPD analysis. Am Pot J 71: 535–546

Kantety RV, Zeng XP, Bennetzen JL, Zehr BE (1995) Assessment of genetic diversity in Dent and Popcorn (*Zea mays* L.) inbred lines using inter-simple sequence repeat (ISSR) amplification. Mol Breed 1: 365–373

Kawchuk LM, Lynch DR, Thomas J, Penner B, Sillito D, Kulcsar F (1996) Characterization of *Solanum tuberosum* simple sequence repeats and application to potato cultivar identification. Am Pot J 73: 325–335

Milbourne D, Meyer R, Bradshaw JE, Baird E, Bonar N, Provan J, Powell W, Waugh R (1997) Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. Mol Breed 3: 127–136

Nagaoko T, Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor Appl Genet 94: 597–602

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

Provan J, Powell W, Waugh R (1996) Microsatellite analysis of relationships within cultivated potato (*Solanum tuberosum*). Theor Appl Genet 92: 1078–1084

Salimath SS, de Oliveira AC, Godwin ID, Bennetzen JL (1995) Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. Genome 38: 757–763

Sosinski B, Douches DS (1996) Using polymerase chain reaction based DNA amplification to fingerprint North American potato cultivars. Hortic Sci 31: 130–133