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Microsatellite analysis of relationships within cultivated potato (*Solanum tuberosum*)

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Abstract The potential of microsatellite markers for use in genetical studies in potato (*Solanum tuberosum*) was evaluated. Database searches revealed that microsatellite sequences were present in the non-coding regions of 24 potato genes. Twenty-two sets of primers were designed and products successfully amplified using 19 primer pairs. These were tested against a panel of 18 tetraploid potato cultivars. Four pairs of primers designed to amplify microsatellites from tomato were also used. Seven (including 2 of the tomato sequences) failed to reveal any variation in the accessions tested. Sixteen primer pairs did reveal polymorphism, detecting between 2 and 19 alleles at each locus. Of these, 3 gave rise to complex band patterns, suggesting that multiple polymorphic loci were being amplified using a single primer pair. Heterozygosity values ranged from 0.408 to 0.921. Phenetic analysis of the derived information allowed a dendrogram to be constructed depicting the relationships between the 18 potato cultivars. The potential of microsatellite markers for genetic analysis and satutory applications in potato is discussed in the context of these results. Furthermore, the potential of 'cross-species amplification' is highlighted as an additional source of microsatellite markers for genetic research in potato.

Key words Genotyping · Microsatellites · Potato · Tomato

Introduction

The development over 10 years ago of restriction fragment length polymorphism (RFLP) analysis demonstrated the viability of studying DNA sequence polymorphisms to construct linkage maps and to examine the genetic relationships between individuals (Botstein et al. 1980; Soller and Beckmann 1983). Since then polymerase chain reaction (PCR)-based marker systems such as randomly amplified polymorphic DNA (RAPDs; Welsh and McClelland 1990; Williams et al. 1990) and sequence-tagged sites (STSs; Olson et al. 1989) have also become widely used due to the relative simplicity of DNA amplification-based methods when compared to RFLP technology (for review see Rafalski and Tingey 1993). More recently, the amplification of highly polymorphic microsatellite sequences has become established as the marker system of choice in studies of mammalian genomes (Wiessenbach et al. 1992; Dietrich et al. 1992; Serikawa et al. 1992; Bishop et al. 1994; Rohrer et al. 1994). These sequences are regions of short, tandemly repeated DNA motifs (generally ≤ 4 bp in length) with an overall length in the order of tens of base pairs that are found dispersed throughout the genomes of all eukaryotes (Hamada et al. 1982; Tautz and Rentz 1984). It is thought that slippage during DNA replication leads to high levels of length polymorphism and amplification of microsatellites using PCR primers flanking the repeated region can be used to exploit the former as highly polymorphic genetic markers (Litt and Luty 1989; Tautz 1989; Beckmann and Soller 1990; Hearne et al. 1992). To date, the assignment of 30 microsatellite loci to the *Arabidopsis thaliana* linkage map (Bell and Ecker 1994) represents the only reported plant-based study on a scale similar to those reported in mammals. Nevertheless, microsatellite polymorphisms have been reported in soybean (Akkaya et al. 1992; Morgante and Olivieri 1993), rice (Wu and Tanksley 1993; Zhao et al. 1993), maize (Senior and Heun 1993), tropical trees (Condit and Hubbell 1991), sunflower (Brunel 1994), grapevine (Thomas and Scott 1993) and barley (Saghai-Maroof et al. 1994). The types of repeats found in plants differs markedly from those found in animals, with $(A \bullet T)_n$ and $(AT \bullet TA)_n$ being the most common (Langercrantz et al. 1993; Wang et al. 1994).

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By far the simplest way to identify microsatellites and obtain information on their flanking sequences to facilitate primer design is to search nucleic acid sequence databases for accessions containing short tandem repeats. In plant species, however, the number of genomic sequences available is still comparatively low. One possible way to overcome the relative lack of available sequence data without embarking on a microsatellite isolation programme was demonstrated by Thomas and Scott (1993) who successfully used grapevine (*Vitis vinifera*) – derived primers to amplify polymorphic microsatellites from other *Vitis* species.

Here we report the amplification of polymorphic microsatellite markers from potato (*Solanum tuberosum*) using primers derived from both potato and tomato (*Lycopersicon esculentum*) genomic DNA sequences and discus their potential for applications in potato genetics.

Materials and methods

Biological material

Genomic DNA was isolated from fresh leaf material using either the CTAB method of Saghai-Maroof et al. (1984) or the method of Edwards et al. (1991). Studies were carried out on the following 18 tetraploid potato cultivars: 'Baillie', 'Centifolia', 'Croft', 'Famosa', 'Foxton', 'Glenna', 'Golden Wonder', 'Majestic', 'Pentland Javelin', 'Red Fife', 'Redskin', 'Rocket', 'Shelagh', 'Shepody', 'Stormont Enterprise', 'Stroma', 'Tahi' and 'Wilja'.

Database searching and primer design

Potato and tomato accessions in the EMBL nucleic acid sequence database were searched for all possible mono-, di- and tri-nucleotide microsatellites using the FASTA programme (Genetics Computer Group). Twenty, 10 and 6 repeats were used to search for mono-, di- and tri-nucleotide repeats, respectively, but the nature of the search also allowed identification of shorter repeats, as well as of imperfect and compound microsatellites. Primers were designed to amplify these repeats using OLIGO (V4.0) or PRIMER (V0.5) and were synthesised in-house on an ABI 391 PCR-Mate oligonucleotide synthesiser. For cross-species amplification, primer annealing sites were chosen within coding regions where possible to maximise the likelihood of sequence conservation.

PCR amplification of microsatellites

Non-radioactive PCR was carried out in a total reaction volume of 10 µl containing 1 × PCR buffer [20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.05% W1], 200 µ*M* dNTPs, 10 pmol each primer, 0.05 U *Taq* DNA polymerase (Gibco BRL) and 20 ng template DNA. For radioactive PCR, 1 µCi[α-^{32}P] dCTP was added to each reaction, and a modified dNTP mixture was used (200 µ*M* dATP/dGTP/dTTP; 5 µ*M* dCTP). All amplifications were carried out on a Perkin/Elmer 9600 thermal cycler using the following parameters: (1) 94°C for 3 min, [T_m] for 2 min, 72°C for 1.5 min × 1 cycle; (2) 94°C for 1 min, [T_m] for 2 min, 72°C for 1.5 min × 29 cycles; (3) 72°C for 5 min, (for T_m values refer to Table 1). Non-radiolabelled products were resolved on 2% agarose gels in 1 × TBE, stained with ethidium bromide and visualised by UV illumination. Radiolabelled PCR products were denatured by the addition of 10 µl stop solution (95% formamide) and heating to 94 °C for 5 min. Samples (2 µl) were then loaded onto 6% polyacrylamide denaturing gels containing 8 *M* urea and separated for 2–4 h at 40 W constant power. Gels were wrapped in cling-film and exposed to X-ray film for 2–16 h without intensification screens.

Cloning and sequencing of PCR products amplified using tomato-derived primers

PCR products were ligated into pGEM-T (Promega) according to the manufacturer's instructions and transformed by electroporation into *E. coli* strain DH5α. Those clones which contained an insert of the expected size after *Spe*I/*Sph*I digestion were sequenced by the Sequenase method (V2.0, USB).

Analysis of results

Results were analysed using the GENSTAT V5.0 (1987) software package. Each band was treated as a unique character with shared absences given equal weighting as shared bands. The degree of similarity between cultivars was calculated according to the number of shared bands (Nei and Li 1979). Heterozygosity values were calculated according to Nei's index, $1-\Sigma[p_i^2]$, where p_i is the frequency of the *i*th phenotype, the phenotype in this case being defined by the pattern observed for a particular individual using a single pair of primers.

Results and discussion

Occurrence of microsatellites and levels of polymorphism

A search of the EMBL database for mono-, di- and tri-nucleotide repeats in potato showed that microsatellites were present in 35 different genes. These were found to be fairly evenly distributed between 5′-untranslated, 3′-untranslated, intron and exon sequences. Eleven were tri-nucleotide repeats present within coding regions, and these were not considered further in this study. Of the remaining 24, 2 were found to be too close to the extreme 5′- or 3′-ends of the published sequences to design a pair of primers. A total of 22 pairs of primers were subsequently synthesised to amplify microsatellites in potato. Three of these failed to generate the expected amplification products. Of the remaining 19, 5 were monomorphic in the 18 cultivars tested, and 3 (STINHWI, STIIKA, STU6SNRN) gave rise to complex band patterns. This is probably due to the amplification of multiple microsatellite-containing loci (for example, in a gene family) using a single pair of primers. The high levels of polymorphism revealed by such multi-locus microsatellite primers make them extremely powerful tools for differentiating between individual cultivars and accessions, and in this study a single primer pair (STIIKA) was able to distinguish all 18 potato cultivars examined.

As microsatellite discovery and development is a time-consuming and expensive exercise, we were particularly interested in the possibility of using simple sequence repeat-containing tomato sequences to expand the repertoire of informative markers in potato (and vice-versa). Thirty-seven microsatellite-containing sequences from tomato were identified in the database searches. Primers were designed to amplify 4 of these. Where possible, primer sequences were derived from exons to maximise the probability that they would be

conserved in potato. Two revealed polymorphism in the 18 cultivars analysed, and 2 were monomorphic. Results obtained with the 16 polymorphic microsatellites are summarised in Table 1. Amplification products from one of the single-locus (STPRINPSG) and one of the multi-locus (STIIKA) primer pairs are shown in Fig. 1.

Between 2 and 19 alleles were found at each of the 16 polymorphic loci, giving rise to heterozygosity values of between 0.408 and 0.921. Average values for number of alleles and heterozygosity were 6.813 and 0.794, respectively. These values are comparable to those reported in similar studies in other plant species (Wu and Tanksley 1993; Bell and Ecker 1994) and demonstrate an obvious advantage of microsatellite analysis over other genetic marker techniques such as AFLPs, RAPDs and RFLPs where average heterozygosity values are generally lower (Powell et al. 1994).

Conservation of microsatellites between potato and tomato

To investigate the basis of the fragment profiles observed, we cloned and sequenced PCR products amplified from tomato sequence-derived primers. Comparison of the sequences obtained from monomorphic (LELAT59G) and polymorphic (LEGAST1) potato products with the corresponding tomato genomic sequence revealed that the monomorphic potato DNA product lacked a microsatellite, whereas the polymorphic product contained a microsatellite of similar composition and location to that found in the tomato gene (Fig. 2). Polymorphism across species is therefore maintained by conservation of the hypervariable microsatellite sequence. The occurrence of vestigial repeats in the form of single motifs in potato [in the case of the LELAT59G product (AT) and (TGCAA) – see Fig. 2a] and the presence of a shorter repeat in the potato LEGAST1 sequence than the corresponding tomato sequence (Fig. 2b) suggests that amplification of the basic motif has occurred to a greater extent in tomato than in potato at these loci (i.e. the potato sequence represents the more primitive state).

Phenetic analysis of relationships between potato cultivars

To examine relationships between the accessions based on the microsatellite results, shared-band analysis in GENSTAT V5.0 (1987) was used to produce the dendrogram shown in Fig. 3. The reasons for the groupings obtained are not obvious from co-ancestry as it is difficult to establish the precise pedigree of the majority of potato cultivars. The estimated similarity between the accessions was 44–81% (Table 2). These similarity levels are almost certainly the result of the high polymorphism revealed by microsatellites at each locus and the approach taken to analyse the data, which involved scoring each allele as a unique character. The latter is particularly relevant because in an autotetraploid outbreeder each individual could contain between one and four different alleles at any one locus. In addition, within the gene pool studied there may be up to n different alleles [in this study, when one only considers the single-locus microsatellites, n ranged from 2 to 9 but may be much higher (e.g. Saghai-Maroof et al. 1994)]. Thus, if we consider an example where 2 cultivars each contain the same two alleles at a specific locus, the first cultivar being triplex for one allele and the second being triplex for the other, by simple matching (the easiest analysis to

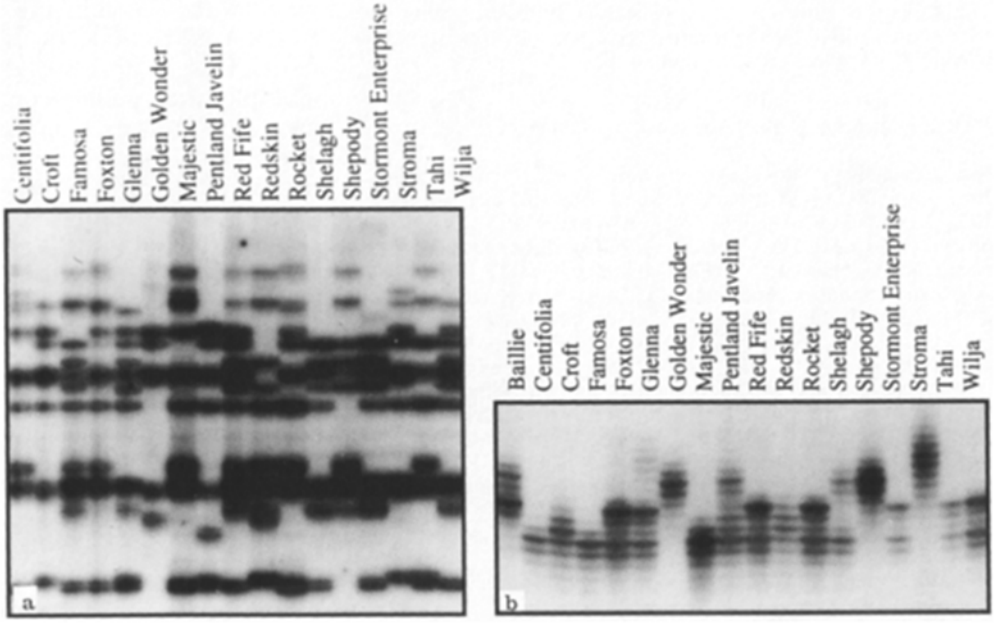

Fig. 1a, b Amplification of microsatellite loci in a panel of 18 tetraploid potato cultivars: **a** STPRINPSG (single locus); **b** STIIKA (multi-locus)

Table 1 Microsatellites used in this study

Name	Gene	Repeat	Location	Primer sequence (5′–3′)	T_m (° C)	Size (bp)	Numbers of alleles	Heterogeneity
STSNRNA10	Potato U1snRNA gene U1-10	$(A)_{19}$	5′-UTR	AGTACTCAGTCAATCAAAG AGGTAAGTATGTTCTCCAG	52	173	5	0.587
STRBCS3	Potato *rbcS3* gene for RUBISCO small subunit	$(A)_{15}$	5′-UTR	CCACAGTGTAATAAGCAT TAGTAACGGGGAAAGACG	50	201	5	0.859
STCPKIN3	Potato cytosolic pyruvate kinase	$(T)_8(G)_5T(G)_9$	Intron III	AAGGAGAGAAGAACATAA CATTGAATAACACCAT	50	168	5	0.881
STPATP1	Potato patatin pseudogene (SB6B)	$(AT)_{22}$	Intron II	TGCAATGTGTCGAACAATCA TAATTGGATAGGTCGGCCTG	56	203	9	0.865
STPOACUTR	Potato *PoAc85* gene for actin	$(AT)_{13}$	3′-UTR	TGTGTTTGTTTTTCTGTAT AATTCTATCCTCATCTCTA	48	276	4	0.829
STPRINPSG	Potato proteinase inhibitor pseudogene	$(TA)_{23}$	3′-UTR	TGTACTGGGGAGCCTCAAAG AATTTTAACCTCGTGACATGGG	60	148	5	0.816
ST13ST	Potato *def4* gene for deficiens analogue	$(AT)_{11}$	Intron V	TATTCCCCCTTCCTACTCAA TCTTCCACATTCCTAACCTG	58	202	7	0.809
STINHWI	Potato wound-inducible proteinase inhibitor I	$(CT)_3TT(CT)_8(AT)_9$	Intron I	GGAGTCAAAGTTTGCTCACATC CACCCTCAACCCCCATATC	60	162	11	0.903
STIIKA	Potato inhibitor IIK	$(T)_{12}(A)_9ATTCTTGTT(TA)_2CA(TA)_7$	Intron	TTCGTTGCTTACCTACTA CCCAAGATTACCACATTC	50	216	19	0.921
STWIN12G	Potato wound-induced genes WIN1 and WIN2	$(TGAAA)_2(ATA)_6$	3′-UTR	TGTTGATTGTGGTGATAA TGTTGGACGTGACTTGTA	48	167	5	0.812
STGBSS	Potato granule-bound starch synthase	$(TCT)_9$	Intron I	AATCGGTGATAAATGTGAATGC ATGCTTGCCATGTGATGTGT	58	138	6	0.878
STAMFWAX	Potato *Amf* locus, waxy locus DNA encoding pseudogene similar to β-1,3-glucanases	$(GTA)_6$	5′-UTR	ATGCCTGTTACGAATAACTCGG CAGCTAACGTGGTTGGGG	58	175	6	0.741
STGLGPB	Potato α-glucan phosphorylase type II (*glgP*)	$(ATT)_5TTT$	5′-UTR	GACACGTTCACCATAAAA AGAAGAATAGCAAAGCAA	48	132	2	0.408
STU6SNRN	Potato U6snRNA	$(TGG)_5$	3′-UTR	GAAGTTTTATCAGAATCC ATCACCTCATCAGCAATC	48	181	9	0.902
LEGAST1	Tomato GAST1	$(TA)_{12}$ and $(TG)_4GT(TG)_5$	Intron I	GTTCTTTTGGTGGTTTTCCT TTATTTCTCTGTTGTTGCTG	54	204	6	0.810
LERBCO	Tomato ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit gene	$(TG)_4(TA)_6TGTA$	Intron I	CATTACCTCCATTGCTA GTCTCGTACTTCTTCAT	48	172	5	0.698

a

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Potato    ATTCGTCATGCTGTTATCCAAAAGGAGCCATTGTGGATCATATTTAAAAG
          |||||||||||||||||||||||||||||||||||||||||||||||||
Tomato    ATTCGTCATGCTGTTATCCAAAAGGAGCCATTGTGGATCATATTTAAAAG

Potato    GGGTATGAACATTAGGTAAGTAGTCAAGATTGTCACACATAATATAT...
          |||||||||||||||||||||||  | | || ||       ||||||
Tomato    GGGTATGAACATTAGGTAAGTACGTACGTTTCTCTATATATATATATGCA

Potato    ..........ATGCAAATAGAATTCAACTTGAAAATGCTCAT.......
                    ||||||||||||||||||  | ||    ||
Tomato    ATGCAATGCAATGCAAATAGAATTCAATGCTCATATATATATATATATAT

Potato    ......TATTTTGAATAGGTTGCACCAGGAGATGATCATGCAGAGTGACA
                | |  ||||||||||||||||||||||||||||||||||||||
Tomato    ATAATGTTTCATGAATACCTTCGACCAGGAGATGATCATGCAGAGTGACA

Potato    AGACGATA
          ||||||||
Tomato    AGACGATA
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b

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Potato    GTTCTTTTGGTGGTTTTCCTGACTCAAAATCAGGTGAGCCCAAATGTTTT
          |||||||||||||||||||||||||||||||||||||||||||||||||
Tomato    GTTCTTTTGGTGGTTTTCCTGACTCAAAATCAGGTGAGCCCAAATGTTTT

Potato    GTGCATCAGTCTATTGTTTTCGACTCGAATTATATATATA.........
          | |||||  ||||||||||||||||||||||||||||
Tomato    GCGCATC..TCTATTGTTTTCGACTCGAATTATATATATATATATATATA

Potato    ......CGTATTGTTGAAAATATATTAAATGATGTGTGTGGTTGTGTGTT
                 ||||||||||||||||||||||||||||||||||||||||||||
Tomato    TATACACGTATTGTTGAAAATATATTAAATGATGTGTGTGGTTGTGTGTT

Potato    CAGGTTTCAAGGGCCAACATTATGCGTGATGAGCAGCAGCAACAACAGAG
          |||||||||||||||||||||||||||||||||||||||||||||||||
Tomato    CAGGTTTCAAGGGCCAACATTATGCGTGATGAGCAGCAGCAACAACAGAG

Potato    AAATAA
          ||||||
Tomato    AAATAA
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Fig. 2a, b Alignments of potato and tomato sequences amplified with **a** tomato LELAT59G primers and **b** tomato LEGAST1 primers

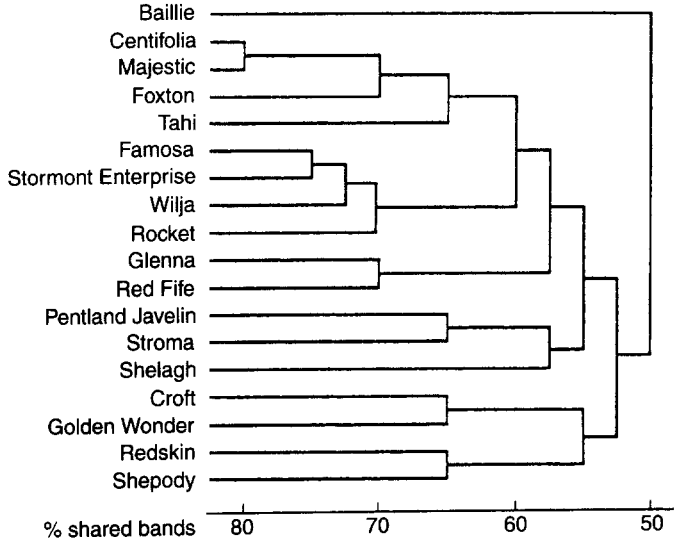

Fig. 3 Dendrogram derived from shared-band analysis in GENSTAT V5.0

describe) both cultivars would be considered identical (regardless of n). In reality, they should be considered only 50% similar. Likewise, the opposite effect can also be obtained. If, for example, the first accession was tetraplex at a locus and the second duplex for the same allele at that locus and simplex for two different alleles (i.e. $n = 3$), then our measure of similarity would be 33%. Again, the true figure would be 50%. Furthermore, if in

Table 2 Similarity matrix (%)

	Baillie	Centifola	Croft	Famosa	Foxton	Glenna	Golden Wonder	Majestic	Pentland Javelin	Red Fife	Redskin	Rocket	Shelagh	Shepody	StormontEnterprise	Stroma	Tahi	Wilja
Baillie	1.00																	
Centifolia	0.56	1.00																
Croft	0.58	0.63	1.00															
Famosa	0.53	0.64	0.57	1.00														
Foxton	0.63	0.70	0.67	0.52	1.00													
Glenna	0.54	0.66	0.57	0.64	0.58	1.00												
Golden Wonder	0.56	0.60	0.67	0.54	0.58	0.52	1.00											
Majestic	0.57	0.81	0.64	0.62	0.71	0.56	0.52	1.00										
Pentland Javelin	0.48	0.68	0.57	0.58	0.50	0.55	0.58	0.67	1.00									
Red Fife	0.56	0.63	0.63	0.64	0.67	0.71	0.54	0.58	0.55	1.00								
Redskin	0.52	0.63	0.63	0.60	0.52	0.49	0.60	0.61	0.54	0.51	1.00							
Rocket	0.54	0.66	0.66	0.70	0.67	0.55	0.55	0.61	0.66	0.63	0.51	1.00						
Shelagh	0.52	0.58	0.53	0.59	0.62	0.56	0.56	0.64	0.66	0.58	0.58	0.59	1.00					
Shepody	0.54	0.55	0.55	0.55	0.44	0.47	0.61	0.56	0.50	0.58	0.67	0.50	0.59	1.00				
Stormont Enterprise	0.59	0.61	0.55	0.75	0.61	0.61	0.52	0.61	0.66	0.71	0.58	0.73	0.71	0.59	1.00			
Stroma	0.54	0.70	0.64	0.52	0.59	0.64	0.58	0.59	0.67	0.64	0.55	0.58	0.56	0.56	0.67	1.00		
Tahi	0.50	0.66	0.63	0.52	0.64	0.60	0.57	0.67	0.66	0.63	0.57	0.66	0.53	0.58	0.58	0.55	1.00	
Wilja	0.56	0.71	0.63	0.72	0.64	0.68	0.61	0.72	0.63	0.63	0.69	0.68	0.66	0.61	0.73	0.53	0.71	1.00

this case $n = 10$, then the similarity value obtained would be 80% (i.e. one shared presence of an allele, seven absences). The conclusion must therefore be that the level of significance attached to the similarity values and phenetic relationships based on simple matching is low. Although it is more difficult to describe, the shared band analysis used here (Nei and Li 1979) also suffers from the same deficiencies. While "weighting" the shared presence of an allele over the shared "absence" may alleviate some of the problems of interpretation due to shared absence of bands, we must conclude that a different set of procedures need to be applied to the phenetic interpretation of microsatellite data in outbreeding autopolyploid species. To our knowledge more appropriate procedures are currently not available. However, different measures of similarity could, for example, be assigned on the basis of the number of repeat unit differences between allelic variants (i.e. a one repeat unit difference would be assigned a higher similarity than a two repeat unit difference). This of course assumes that repeat length variation occurs one unit at a time, which may not necessarily be the case (Dirienzo et al. 1994). In addition, methods for estimating "allele dosage" at a given locus by, for example, comparison of the signal strength of each allelic PCR fragment would help in more accurately determining the genotype of the accessions under study. By way of caution, this approach relies on "quantitative" PCR, which may not necessarily operate symmetrically, even for allelic variants within the same tube. Nevertheless, the presence of multiple alleles at microsatellite loci makes them at the very least a powerful tool for genotyping individuals if not for determining phylogeny. This has been illustrated above where a single microsatellite was sufficient to discriminate between all 18 potato cultivars studied. The inclusion of further loci with comparable heterozygosity values may ultimately allow for the identification of almost any cultivar using only 3 or 4 pairs of microsatellite primers. The tagging of these primers with various fluorescent molecules should facilitate the analysis of multiple loci on a single gel by automated data analysis techniques (Thomas et al. 1994). The implications for statutory applications such as determination of seed purity or for protecting plant breeders' rights are therefore clear, especially given the robustness and simplicity of the approach. For genetic linkage studies, it is difficult to predict whether microsatellites will open up the possibility of examining tetraploid crosses as the genetical complexities of tetrasomic inheritance remain unchanged. In diploid crosses where the genetics are much easier to interpret, microsatellites should provide a more user-friendly and equally informative alternative to RFLPs. In addition, the design of primers to amplify microsatellites based on genomic sequence from a close relative of the plant under study (in this case, using tomato-derived primers in potato) should provide many extra polymorphic markers for genome research. This perhaps not unexpected observation circumvents the resource-demanding development phases implicit in this type of research. A search of the EMBL and GenBank nucleic acid databases for microsatellites in other solanaceous species (tomato, pepper, aubergine and tobacco) has revealed that they are present in a large number of genes (Table 3). With respect to maximal homology, the careful design of primers from related species may provide a rich source of polymorphic microsatellite markers for applications in genomic analysis within a genus.

Table 3 Occurrence of microsatellites in solanaceous species

Species	Number of gene sequences in database	Number of microsatellites	Percentage of genes containing microsatellites
Potato	107	30	28
Tomato	154	37	24
Tobacco	280	47	17
Pepper	5	0	0

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