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Microsatellites for cultivar identification in *Pelargonium*

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Abstract We have isolated and characterised microsatellite loci from *Pelargonium* sp. to explore the potential of these markers for cultivar identification. Small-insert libraries from a zonal (*Pelargonium x hortorum* cv. Isabell) and an ivy-leaved variety (*P. peltatum* cv. Guenievre gergue) were enriched for d(AG), d(AC), d(CAA), d(GAA) and d(GATA) repeats. Of 141 positive clones sequenced, 133 contained a microsatellite. Primers for PCR amplification were designed to the flanking regions of 57 microsatellites, resulting in interpretable amplification products of the expected size for 29 loci. Seventeen primer pairs amplifying 18 loci were used to fingerprint 44 di- and tetra-ploid *Pelargonium* accessions representative of commercially available varieties. Multilocus genotypes obtained at 3 loci distinguished among all accessions, except for three known flower colour sports and a fourth, phenotypically very similar, variety. Allelic composition was also identical within two other sport ‘families’ typed at the same 18 loci. UPGMA and principal co-ordinate analysis of pairwise distance matrices derived from PCR amplification patterns revealed four distinct assemblages. The first group consist-

ed of tetraploid *P. x hortorum* varieties; a second group contained diploid *P. x hortorum*, a third, tetraploid *P. peltatum* accessions, while a fourth, very distinct, group consisted solely of diploid *P. peltatum* varieties. Polymorphism in *P. peltatum* was equal or greater than in *P. x hortorum* at 17 of the 18 loci, indicating that the analysed *P. peltatum* varieties form a genetically more variable array.

Keywords Cultivar identification · Geranium · *Pelargonium* · Simple sequence repeats · Protected varieties

Introduction

Cultivated *Pelargonium* species, more generally known as ‘house geraniums’, are drought resistant plants, popular for their ornamental qualities. Pelargoniums have been bred for approximately 200 years, and most commercially available varieties originate from South Africa. The genus *Pelargonium* comprises over 250 species in 13–17 sections (e.g. Van der Walt and Vorster 1988; Van der Walt and Van Zyl 1988). Of these, sections *Ciconium*, *Dibrachya* and *Pelargonium* contain the bulk of the commercially successful species. The two most commonly cultivated pelargoniums in Europe and North America are known as ‘zonal’ and ‘ivy-leaved’. Zonal cultivars (*P. x hortorum* Bailey; section *Ciconium*) are mostly hybrids, derived from *P. zonale* and *P. inquinans*, whereas ‘ivy-leaved’ cultivars were derived from crossings among various subspecies of *P. peltatum* Horn, section *Dibrachya*. These cultivated varieties are either diploid with a total of 18 chromosomes, or tetraploid with 36 chromosomes.

New *Pelargonium* varieties are constantly generated through selection, crossing and by spontaneous mutation known as ‘sporting’. Once generated, these new varieties are propagated as vegetative clones, often with reduced fertility. About 280 ‘protected’ *Pelargonium* varieties are currently registered, and this number is expected to increase. *Pelargonium* breeding has developed into an in-

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dustry of substantial commercial importance, part of which is derived from royalties paid to breeders of protected varieties. A considerable amount of this revenue is lost annually due to fraudulent propagation. The traditional method of detecting such illegal propagation involves the culturing of controversial varieties for 1–2 years under controlled conditions. The cost and time expenditure of these tests are prohibitive, which means that at present fraud remains largely undetected.

Molecular genetic markers may improve this situation in several respects: Firstly, they can facilitate the detection of fraudulent propagation. Secondly, genetic markers may significantly speed up the DUS (distinctness-uniformity-stability) test, which describes and assesses candidate varieties for 'protected' status (Löschner 1992). Thirdly, genetic markers have the potential to identify 'essentially derived' varieties, such as sports and transgenics. While essentially derived varieties are phenotypically different from their 'parent', they should be genotypically almost identical. Finally, the availability of genetic markers may permit the 'tailoring' of new varieties by marker-assisted selection.

The potential of genetic markers for cultivar identification and fraud detection in *Pelargonium* has prompted several studies in this direction. Renou et al. (1997) used random amplified polymorphic DNAs (RAPDs; Williams et al. 1990) to investigate genetic variation among *Pelargonium* varieties and hybrids. The 34 accessions examined clustered into three distinct groups. The first group comprised diploid and tetraploid zonal pelargoniums; the second group contained tetraploid ivy-leaved *P. peltatum* varieties only; while the third consisted of 5 diploid ivy-leaved somatic mutants that were indistinguishable by RAPD markers. Hybrids were positioned between their respective parents. However, unequivocal identification of varieties was not achieved. Starman and Abbitt (1997) reported the successful discrimination of individual *Pelargonium* cultivars by the ASAP technique (arbitrary signatures from amplification profiles; Caetano-Anollés and Gresshoff 1996), but only 8 varieties were examined. More recently, Lesur et al. (1999) evaluated seven different marker techniques for their usefulness in the genetic discrimination of 23 *Pelargonium* varieties. Results of this preliminary study indicated that RAPD, microsatellite and AFLP (amplified fragment length polymorphism; Vos et al. 1995) markers were sufficiently variable to merit further study.

Microsatellites are hypervariable single-locus markers that have previously been shown to be highly informative in varietal identification, for example in wheat (*Triticum aestivum*; Plaschke et al. 1995; Bryan et al. 1997), apples (*Malus x domestica*; Guilford et al. 1997; Gianfranceschi et al. 1998; Hokanson et al. 1998), tomatoes (*Lycopersicon esculentum*; Bredemeijer et al. 1998) and kiwifruit (*Actinidia chinensis*; Weising et al. 1996; Huang et al. 1998). In the investigation presented here we assessed the power of microsatellite markers to identify individual accessions among a representative set of commercially available *Pelargonium* varieties.

Materials and methods

Plant material

Plasmid libraries were constructed from tetraploid *P. peltatum* cv. Guenievre guergue and tetraploid *P. x hortorum* cv. Isabell, respectively. Forty-three cultivated *P. x hortorum* and *P. peltatum* varieties and one wild sub-species, *P. peltatum* ssp. *lateripes*, were used to assess microsatellite polymorphism (Table 1). The analysed material was selected for its representativity of varieties commercially available across continental Europe. In addition, nine somatic mutant varieties belonging to two families of sports were examined for allelic variation (Table 2). The relative abundance of different microsatellite motifs was determined for a sub-set of 12 varieties from Table 1: WES, VIO, BIC, REN, FEE, ISA, GRP, SCH, GUE, MOR, RED, ROI 1, and DIB.

DNA extraction and Southern analysis

Genomic DNA was extracted from leaf tissue using the cetyl trimethylammonium bromide (CTAB) procedure of Weising et al. (1995) with the following modifications. Up to 3 g of fresh leaf material was ground in liquid nitrogen and incubated for 15 min at 55°C in (3 ml per gram tissue of) pre-warmed 2× CTAB buffer with 1% β-mercaptoethanol. Following ethanol precipitation, RNase A was added to the redissolved DNA. Additional purification steps such as polysaccharide precipitation (Michaels et al. 1994), ammonium acetate treatment and/or phenol-chloroform extraction were performed as required. Final DNA preparations were dissolved in 10 mM TRIS-HCl pH 8, 1 mM EDTA.

The relative abundance of different microsatellite motifs in *Pelargonium* was estimated by Southern blot analysis. Genomic DNA (2 µg) from 8 *P. x hortorum* and 4 *P. peltatum* varieties was restricted with *TaqI*, the fragments were then separated by agarose electrophoresis and immobilised on a nylon membrane using the standard Southern protocol (Sambrook et al. 1989). Oligonucleotides consisting of microsatellite motifs common in plant genomes [d(CA)_n, d(GA)₁₂, d(AAG)₆, d(ATG)₆, d(GATA)₅; Lagercrantz et al. 1993; Wang et al. 1994] were 3'-endlabelled with digoxigenin (Boehringer Mannheim) and hybridised with the membrane at 42°C according to the protocols given by Boehringer Mannheim and CIMMYT (Hoisington et al. 1994).

Construction of small-insert libraries and enrichment for microsatellites

Two small-insert genomic libraries were set up using different enrichment techniques. For the *P. peltatum* library, the magnetic bead technique described by Kijas et al. (1994) was modified as follows: genomic DNA from *P. peltatum* cv. Guenievre guergue was digested with *Sau3AI*. DNA fragments of 400–800 bp were isolated by purification from agarose gels. A single round of subtraction hybridisation with a mixture of biotinylated d(AG)₁₂, d(AAG)₆ and d(GATA)₅ oligonucleotides was carried out as in Kijas et al. (1994), and the enriched library was ligated into *Bam*HI digested, dephosphorylated pUC18 (Pharmacia). Following transformation into supercompetent XL1-Blue MRF' cells (Stratagene), colonies were grown on SOB agar overnight. Positive colonies, identified by blue/white screening, were transferred to gridded 220×220-mm petri dishes and grown over-night for secondary screening by colony hybridisation with 3' digoxigenin endlabelled probes. Colonies thus identified as containing microsatellites were directly polymerase chain reaction (PCR)-amplified using universal M13 primers (Rafalski et al. 1996) to determine insert length. Plasmid DNA from clones with a minimum insert size of 300 bp was extracted, column-purified (Qiagen) and analysed by automated sequencing.

The second small-insert library was constructed from *Pelargonium x hortorum* cv. Isabell following Fischer and Bachmann (1998) with several modifications. Genomic DNA was digested

Table 1 Plant material. *Pelargonium* accessions representative of cultivars commercially available on the European market. Likelihoods refer to the probability of obtaining a particular allele pheno-

type at three loci (Phi21, Phi45, Phi49) within the *P. x hortorum* and *P. peltatum* groups, respectively

Variety	Code	Species	Chromosome no.	Set	Likelihood
Westphalen ^a	WES	<i>Pelargonium x hortorum</i>	2n=18	1	6.75×10 ⁻⁵
Regat Sun ^a	REG	<i>P. x hortorum</i>	2n=18	1	1.46×10 ⁻⁶
Violine ^a	VIO	<i>P. x hortorum</i>	2n=18	1	2.03×10 ⁻⁵
Bicolor ^a	BIC	<i>P. x hortorum</i>	2n=18	1	2.95×10 ⁻⁵
Renard Bleu ^a	REN	<i>P. x hortorum</i>	2n=18	1	9.91×10 ⁻⁹
Féerie Orange ^a	FEE	<i>P. x hortorum</i>	2n=18	1	5.40×10 ⁻⁶
Cahors potcah ^b	CAH	<i>P. x hortorum</i>	4n=36	1	4.27×10 ⁻⁴
Biarritz potbia ^b	BIA	<i>P. x hortorum</i>	4n=36	1	1.55×10 ⁻³
Rosecrystal ^c	ROS	<i>P. x hortorum</i>	4n=36	1	4.34×10 ⁻⁴
Isabell ^c	ISA	<i>P. x hortorum</i>	4n=36	1	3.59×10 ⁻⁴
Grand Prix ^d	GRP	<i>P. x hortorum</i>	4n=36	1	1.94×10 ⁻⁴
Schöne Helena ^d	SCH	<i>P. x hortorum</i>	4n=36	1	1.80×10 ⁻⁴
Sidonie ^c	SID	<i>P. x hortorum</i>	4n=36	1	9.56×10 ⁻⁵
Guenievre gergue ^b	GUE	<i>Pelargonium peltatum</i>	4n=36	1	2.68×10 ⁻⁵
Morgane germorg ^b	MOR	<i>P. peltatum</i>	4n=36	1	3.32×10 ⁻⁵
Granatit ^c	GRA	<i>P. peltatum</i>	4n=36	1	6.80×10 ⁻⁵
Red Blizzard ^d	RED	<i>P. peltatum</i>	4n=36	1	2.23×10 ⁻⁵
Amethyst 96 ^d	AME	<i>P. peltatum</i>	4n=36	1	6.13×10 ⁻⁵
Roi des Balcons rouge ^b	ROI 1	<i>P. peltatum</i>	2n=18	1	1.29×10 ⁻⁵
Roi des Balcons rosé ^b	ROI 2	<i>P. peltatum</i>	2n=18	1	1.29×10 ⁻⁵
Roi des Balcons lilas ^b	ROI 3	<i>P. peltatum</i>	2n=18	1	1.29×10 ⁻⁵
Ville de Dresden ^c	DRE	<i>P. peltatum</i>	2n=18	1	5.69×10 ⁻⁷
<i>P. lateripes</i> ^a	DIB	<i>P. p. ssp. lateripes</i>	2n=18	1	1.03×10 ⁻⁶
Glacis ^c	GLA	<i>P. x hortorum</i>	4n=36	2	2.13×10 ⁻⁵
Penve ^c	PEN	<i>P. x hortorum</i>	4n=36	2	3.07×10 ⁻³
Wendy ^c	WEN	<i>P. x hortorum</i>	4n=36	2	1.03×10 ⁻³
Bergpalais ^c	BER	<i>P. x hortorum</i>	4n=36	2	8.49×10 ⁻⁴
Tango ^d	TAN	<i>P. x hortorum</i>	4n=36	2	1.42×10 ⁻⁶
Kardino ^d	KAR	<i>P. x hortorum</i>	4n=36	2	3.73×10 ⁻⁵
Tiffany ^d	TIF	<i>P. x hortorum</i>	4n=36	2	1.24×10 ⁻³
Atlantis ^d	ATL	<i>P. x hortorum</i>	4n=36	2	4.18×10 ⁻⁴
Deauville ^b	DEA	<i>P. x hortorum</i>	4n=36	2	1.77×10 ⁻⁵
Angers ^b	ANG	<i>P. x hortorum</i>	4n=36	2	6.29×10 ⁻⁴
Cannes ^b	CAN	<i>P. x hortorum</i>	4n=36	2	7.38×10 ⁻⁴
Dijon ^b	DIJ	<i>P. x hortorum</i>	4n=36	2	2.11×10 ⁻³
Evian ^b	EVI	<i>P. x hortorum</i>	4n=36	2	6.53×10 ⁻⁵
Paul Jovet ^b	JOV	<i>P. x hortorum</i>	4n=36	2	2.01×10 ⁻⁴
Purpalit ^c	PUR	<i>P. peltatum</i>	4n=36	2	6.11×10 ⁻⁴
Coralit ^c	COR	<i>P. peltatum</i>	4n=36	2	1.88×10 ⁻⁴
Evka ^c	EVK	<i>P. peltatum</i>	2n=18	2	1.29×10 ⁻⁵
Luna 98 ^d	LUN	<i>P. peltatum</i>	4n=36	2	5.37×10 ⁻⁶
Molina ^d	MOL	<i>P. peltatum</i>	4n=36	2	1.33×10 ⁻⁵
Blue Blizzard ^d	BLI	<i>P. peltatum</i>	4n=36	2	9.16×10 ⁻⁵
Viviane 97 ^b	VIV	<i>P. peltatum</i>	4n=36	2	4.03×10 ⁻⁵

^{a-d} Superscripts indicate supplier: ^a INRA, France; ^b Technigéra, France; ^c PAC Elsner, Germany; ^d Fischer, Germany

with *MluI*, and restriction fragments were ligated to adaptors containing an internal *MluI* site. Purified ligation products were heat-denatured and hybridised with biotinylated oligonucleotides, either with a pooled d(CA)₁₀, d(CAA)₈ and d(GAA)₈ (hybridisation at 74°C) or with d(GA)₁₀ alone (hybridisation at 60°C). Enriched DNA preparations were concentrated by Microcon-30 spinfilters and PCR-amplified with adaptor-specific primers. An aliquot of the resulting PCR products was subjected to a second round of enrichment under identical conditions. Final PCR products were restricted with *MluI*, ligated into the *BssHII* site of a dephosphorylated pSK vector (Stratagene) and used to transform *E. coli* DH5α cells by electroporation. Positive clones were identified by colony PCR with vector-specific primers and subsequent Southern hybridisation with γ-[³²P]-endlabelled oligonucleotides d(CA)₈, d(GA)₈, d(CAA)₅ or d(GAA)₅. PCR products showing a clear hybridisation signal were re-amplified, column-purified (Qiagen) and analysed by automated sequencing.

PCR

Primers for PCR amplification were designed to microsatellite flanking regions using the programme PRIMER3 (Rozen and Skaletzky 1996). PCR amplification of the 18 loci analysed for this study was robust and reliable, regardless of the type of thermal cycler or the manufacturer of *Taq* polymerase employed, as long as primer-specific annealing temperatures were utilised (Table 3). We give one successful protocol here; variants can be obtained from the authors on request.

Reaction mixes comprised 20 ng of DNA template, 67 mM Tris-HCl (pH 8.8), 16 mM ammonium sulfate, 0.01% Tween 20, 2.0 mM MgCl₂, 0.2 mM dNTP, 0.5 μM of each primer, and 0.025 U/μl *Taq* DNA polymerase (Biotherm) in a total volume of 25 μl. After denaturation at 95°C for 2 min, the reaction was cycled for 30 times through a 94°C denaturing step for 25 s, an annealing step at the primer specific temperature (Table 3) for 45 s and a

Table 2 Plant material, set 3: somatic mutant *Pelargonium* varieties from three sport 'families'. ROI 1, 2 and 3 were also part of set 1 (Table 1)

Somatic mutant	Code	Species	Chromosome no.	Set
Roi des Balcons rouge ^b	ROI 1	<i>P. peltatum</i>	2n=18	3
Roi des Balcons rosé ^b	ROI 2	<i>P. peltatum</i>	2n=18	3
Roi des Balcons lilas ^b	ROI 3	<i>P. peltatum</i>	2n=18	3
Ingre ^a	ING	<i>P. peltatum</i>	4n=36	3
Blanche Roche ^a	BRO	<i>P. peltatum</i>	4n=36	3
Lachskönigin ^c	LAK	<i>P. x hortorum</i>	4n=36	3
Sport v03-1 ^c	LAK 1	<i>P. x hortorum</i>	4n=36	3
Sport v03-2 ^c	LAK 2	<i>P. x hortorum</i>	4n=36	3
Sport v03-3 ^c	LAK 3	<i>P. x hortorum</i>	4n=36	3
Sport v03-5 ^c	LAK 5	<i>P. x hortorum</i>	4n=36	3
Sport v03-6 ^c	LAK 6	<i>P. x hortorum</i>	4n=36	3
Sport v03-7 ^c	LAK 7	<i>P. x hortorum</i>	4n=36	3

^{a-c} Superscripts indicate supplier: ^a INRA, France; ^b Technigéra, France; ^c PAC Elsner, Germany

Table 3 Characteristics of primer pairs amplifying 29 polymorphic microsatellite loci in *Pelargonium* sp. (*PpG* loci cloned from *P. peltatum* Guenievre gergue. *PhI* loci cloned from *P. x hortorum* Isabell. *annealing temp* primer annealing temperatures in degrees Celsius. *nd* not determined). Numbers of phenotypes refer the 44 *Pelargonium* accessions listed in Table 1

Locus	Motif	Annealing temp.	Allele phenotypes	Locus	Motif	Annealing temp.	Allele phenotypes
PpG4 ^a	(TC) ₁₇	52	15	PhI29	(TC) ₇ A(CT) ₈ A(TC) ₄	52	nd
PpG13 ^a	(CA) ₁₈	52	19	PhI30	(AC) ₁₃ T(CA) ₄	52	nd
PpG14 ^a	(GA) ₂₁ GT(GA) ₂₀ TATAGAC(GA) ₂₂	57	4/18	PhI39 ^a	(CTT) ₂₅	52	8
PpG20 ^a	(GA) ₃₀	52	13	PhI40	(GTT) ₄ (GCT) ₆ (GTTGCT) ₄	55	nd
PpG58 ^a	(GA) ₂₃	52	11	PhI45 ^a	(CTT) ₃₁	52	17
PhI02	(GT) ₅ (GA) ₉ GC(GA) ₃	52	nd	PhI48 ^a	(CAA) ₈ ACATT (CA) ₆ (CT) ₂	52	11
PhI12 ^a	(GA) ₁₁	52	8	PhI49 ^a	(GAA) ₁₃ T(GAG) ₂ (AAG) ₂ GA (GAA)GA	52	23
PhI13 ^a	(GC) ₅ N ₂₀ (CT) ₇ C ₄ (CT) ₅	52	10	PhI50	(GTT) ₆	52	nd
PhI17 ^a	(GA) ₅ AA(GA) ₃ N ₃₃ (GA) ₁₁ N ₃₉ (GA) ₇	52	7	PhI55	(GT) ₁₁ (CT) ₂ (GT) ₅	49	nd
PhI21 ^a	(CA) ₁₄	52	17	PhI63 ^a	(GTT) ₈	52	6
PhI24 ^a	(CT) ₇ T ₂ (CT) ₅ (CA) ₇ N ₇₃ (CT) ₁₀ CACT(CA) ₈	49	4	PhI64	(CAA) ₁₀ N ₁₂₀ (CAA) ₅	52	nd
PhI26 ^a	(GT) ₁₂	52	6	PhI66	(CT) ₁₅	52	nd
PhI27	(GT) ₅ (GCGT) ₃ (GT) ₅ N ₂₀ (AG) ₁₇	52	nd	PhI67	(CAACA) ₅ N ₁₇₈ (CTT) ₉	52	nd
PhI28 ^a	(CA) ₁₂	52	6	PhI68	(GAA) ₁₃	52	nd

^a The set of 17 primer pairs used to genotype the entire set of plant material

65°C extension step for 60 s. A final 7 min extension at 65°C concluded the reaction. PCR products were separated either on 2% agarose or 4% Metaphor gels (Flowgen) and visualised by ethidium bromide staining under UV light. For exact sizing and large-scale allele screening, PCR products were mixed with formamide denaturing buffer (Sambrook et al. 1989), heat-denatured and separated by electrophoresis on 6% denaturing polyacrylamide gels (Sambrook et al. 1989). Bands were visualised either by autoradiography or by silver staining (Promega Silver Sequence System). For radioactive PCRs, dCTP or dATP was reduced to 0.02 mM, and an additional 0.06 µl of α-dCTP³² or α-dATP³² were added. Sequencing reactions of pUC18, M13mp18 or pGem 3f(z)+ were loaded alongside microsatellite amplification products to facilitate allele sizing.

Statistical analysis

Although microsatellites generate co-dominant markers, problems can arise during the identification of polyploid genotypes, and the assignment of complete genotypes has to rely on band intensities. Allelic intensity differences in our PCR products were not sufficiently reliable to obtain complete genotypes for the tetraploid heterozygotes observed in *Pelargonium*. Banding patterns observed at a particular locus were therefore recorded as a presence/absence matrix and are referred to as 'allele phenotypes'.

Allele phenotypes from 44 varieties scored at 3 microsatellite loci (PhI21, PhI 45, PhI 49) were analysed with principal co-ordinate analysis (PCO), available in the programme NTSYSPC (Rohlf 1998). Allele phenotypes from 44 varieties scored at 18 microsatellite loci were analysed by cluster analysis with the unweighted pair group method using arithmetic averages (UPGMA). For the latter, pairwise Euclidian distances were first calculated with the

programme RAPDIST 1.04 (Armstrong et al. 1994) and then transferred into PHYLIP v3.57c (Felsenstein 1995) for clustering.

The likelihood of obtaining a particular allele phenotype with 3 primer pairs (PhI21, PhI45, PhI49) was calculated by establishing the frequency of alleles observed in cultivars per locus. The frequencies of alleles characterising a particular accession were then multiplied across the 3 loci. As the phenotypic classification of pelargoniums into either zonal or ivy-leaved is easily possible, likelihoods of obtaining phenotypes were calculated within the *P. x hortorum* and *P. peltatum* groups, respectively.

Results

Abundance of microsatellite motifs

The intensity of patterns generated after Southern hybridisation of microsatellite probes to genomic DNA of 12 zonal and ivy-leaved *Pelargonium* accessions was compared after identical periods of exposure to X-ray film. This revealed that of the five motifs used as probes, d(GA) loci were most abundant, followed by d(CA) and d(GATA). The two trimeric motifs d(AAG) and d(AGT) were least common (results not shown).

Isolation and characterisation of microsatellite loci

Small-insert genomic libraries enriched for microsatellites were constructed from both *P. x hortorum* and *P. peltatum*, using two methods based on subtraction hybridisation and affinity capture. Enrichment of *AluI*-digested *P. x hortorum* cv. Isabell DNA for the presence of d(CA), d(GA), d(CAA) and d(GAA) repeats yielded 86 positives out of 431 randomly picked clones. Performing two rounds instead of one round of selection increased the average efficiency of enrichment more than three-fold, i.e. from 9% to more than 30%. Although a different cloning approach was followed for the *P. peltatum* library, the efficiency of single round of enrichment was in a similar range, with 249 positive signals after screening of 3940 colonies (6.3%). A second round of enrichment was not attempted for *P. peltatum*. A total of 141 clones were selected for sequence determination (71 from *P. x hortorum*, 70 from *P. peltatum*). With the exception of 8 clones, all contained 1 or more microsatellites. Duplicates were quite common, with 1 clone found in 20 copies, another in 4, and a further 6 clones in 2–3 copies. Despite the addition of excess adapter during the enrichment procedures, several fragment concatenates were identified by the presence of internal *AluI* or *Sau3AI* restriction sites.

Level of polymorphism

Fifty-seven primer pairs were designed (44 in *P. x hortorum*, 13 in *P. peltatum*), 32 of which produced interpretable banding patterns of the expected size range. Twenty-eight primer pairs produced polymorphic patterns on

sequencing gels and were selected for further study. One primer pair (PpG14) directed amplification at 2 polymorphic loci. The unexpectedly amplifying second locus was shorter and less polymorphic than the originally cloned locus. Characteristics of the 29 loci are compiled in Table 3. Sequences of primer pairs are available from the authors on request. Seventeen primer pairs amplifying 18 loci (indicated in Table 3) were selected for genotyping of the full set of di- and tetra-ploid accessions. These primer pairs revealed between 2 and 11 alleles, and a maximum of 23 'allele phenotypes' from a single primer pair (PhI45). A total of 115 alleles were identified. No variation was observed between the three 'Roi des Balcons' flower-colour mutants, and a fourth diploid *P. peltatum* accession, Evka. Allelic composition was also identical within 2 other sport families analysed at the same 18 loci (Table 2). Observed heterozygosity for the six diploid *P. x hortorum* plants tested ranged from 0% (PhI26, PhI63) up to 100% (PhI21), depending on the locus. A more precise heterozygosity estimate was not attempted in the frame of this study, because of difficulties in assigning genotypes in tetraploids.

Averaged over 42 accessions (excluding two of the three Roi des Balcons somatic mutants), zonal *Pelargonium* varieties had 4.0 alleles per locus with an average length of 163 bp, whereas ivy-leaved varieties had 5.2 alleles per locus with an average length of 155 bp. Individual alleles were often separated by steps of 2 bp (for dinucleotide repeats) or 3 bp (for trinucleotide repeats), which is consistent with a variable number of basic repeat units. However, larger or smaller size differences were also observed, suggesting that other types of sequence variation are also involved in allelic diversity.

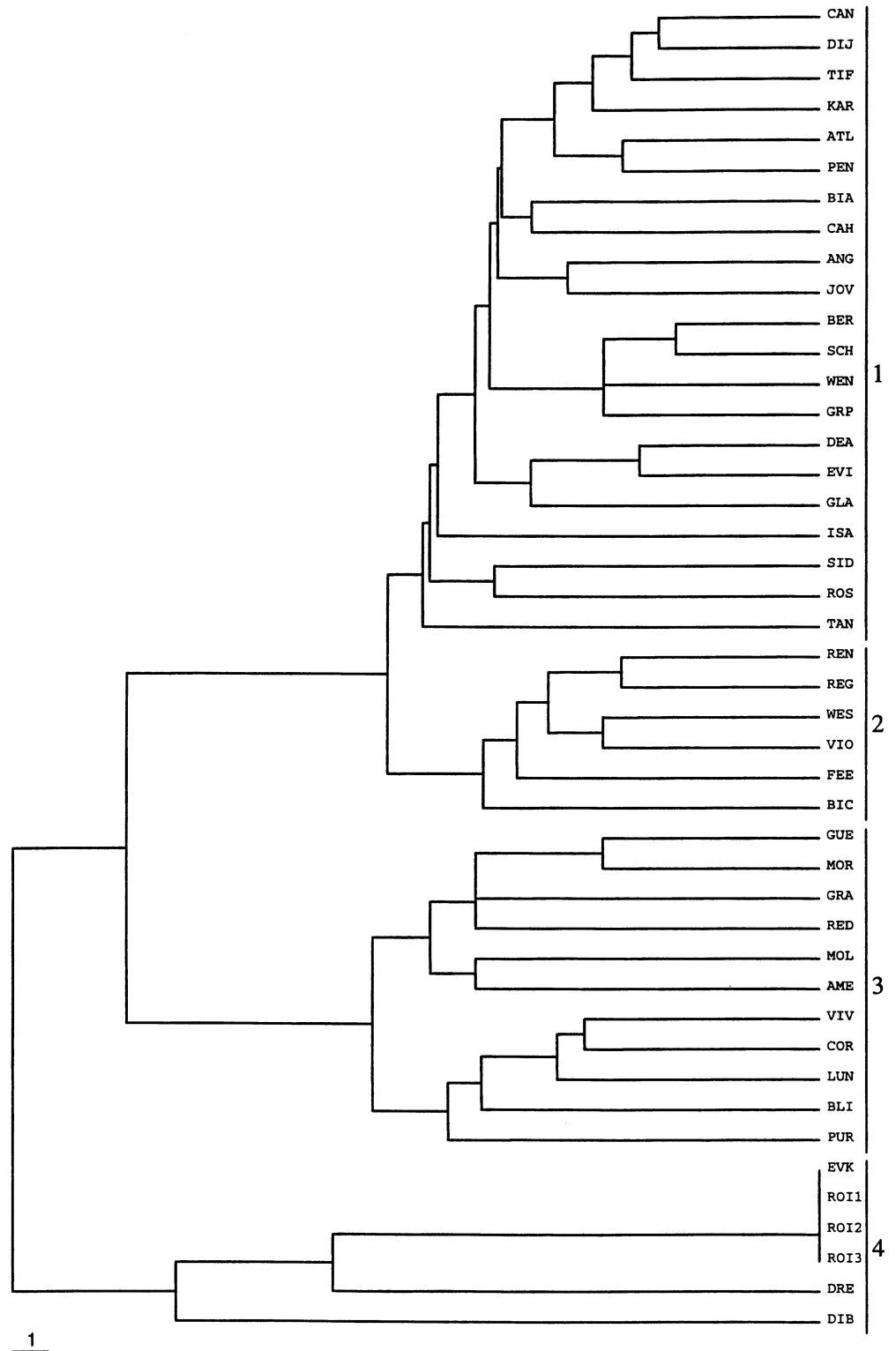
The likelihood of obtaining a particular allele phenotype within either *P. peltatum* or *P. x hortorum* accessions ranged from 1.03×10^{-3} to 9.91×10^{-9} (Table 1).

Relatedness of cultivars and variety identification

UPGMA clustering was used to describe genetic variation and relatedness among the 44 accessions. The UPGMA phenogram (Fig. 1) revealed four distinct groups. The first group comprised all 21 tetraploid *P. x hortorum* accessions. These were most closely related to a second cluster, formed by the six diploid *P. x hortorum* cultivars analysed. The third assemblage consisted of all 11 tetraploid *P. peltatum* varieties, while the final group contained the six diploid *P. peltatum* varieties. Whereas the *P. x hortorum* accessions were closely associated, diploid and tetraploid *P. peltatum* accessions each formed very distinct clusters. As the three somatic mutants (ROI 1–3) possessed the same alleles for all loci, they could not be resolved in the phenogram. Surprisingly, allele phenotypes scored for a fourth diploid *P. peltatum* accession, Evka, were identical to those of the three somatic mutants. This variety therefore also occupied the same position in the UPGMA phenogram.

PCO analysis revealed that 29 alleles, derived from loci PhI21, PhI45 and PhI49, were sufficient to distin-

Fig. 1 Phenogram of 44 *Pelargonium* cultivars of combined test sets 1 and 2 (Table 1) based on shared microsatellite alleles at 18 loci revealed by 17 primer pairs. Numbers correspond to tetraploid *P. x hortorum* (1), diploid *P. x hortorum* (2), tetraploid *P. peltatum* (3) and diploid *P. peltatum* (4). Relative genetic distance is indicated by the horizontal bar



guish among all cultivars bar the somatic mutants and the diploid *P. peltatum* accession Evka (results not shown). The first two principal factors accounted for 19% and 12.2% of the variance in presence and absence of alleles, respectively. In contrast to the four groups identified by UPGMA analysis of 18 loci, PCO analysis

of 3 loci did not permit us to distinguish between diploid and tetraploid *P. x hortorum* varieties. *P. x hortorum* varieties grouped closely together, while patterns for *P. peltatum* varieties were less cohesive. The grouping of tetraploid *P. peltatum* varieties was mainly defined by factor two, whereas 5 diploid varieties fell into a distinct

cluster, largely defined by factor one. The wild species *peltatum* ssp. *lateripes* occupied an isolated position between tetraploid and diploid *P. peltatum* varieties.

Discussion

We report here the first characterisation of microsatellite loci in *Pelargonium* sp. According to both Southern analysis and yield of positive colonies during cloning, the dimeric motifs d(AG) and d(AC) were more abundant in *Pelargonium* than the tri- and tetrameric motifs. This is consistent with observations in other plant species (e.g. Lagercrantz et al. 1993; Wang et al. 1994).

Cloning efficiency following a single round of enrichment in both *P. peltatum* and *P. x hortorum* libraries was relatively low when compared with rates observed in other small-insert libraries using a similar technique. Libraries from citrus (Kijas et al. 1994), onion (Fischer and Bachmann 1998), kiwifruit (*Actinidia chinensis*; Huang et al. 1998) and field bean (*Vicia faba*; Koblizkova et al. 1998) all yielded positive clones in excess of 20%. In *Pelargonium*, this figure was only achieved after a second round of enrichment. This suggests either that microsatellites are relatively rare in *Pelargonium* or that for unknown reasons the enrichment procedures were less efficient in our hands. A surprising number of microsatellite loci were cloned more than once, in particular in the library generated from *P. peltatum*. The occasional occurrence of duplicate clones is known from other microsatellite-enriched libraries and is most probably a consequence of the PCR step after affinity capture. Several chimaeric clones were observed, in particular in the library from *P. x hortorum*. Such chimaeric concatenates generated during the initial restriction/ligation step were easily identified by the presence of internal *AluI*- or *Sau3AI* restriction sites. Another type of chimaera may be formed during the PCR step of the enrichment cloning procedure (discussed e.g. in Koblizkova et al. 1998). Such chimaeras usually remain undetected and may have been responsible for the failure of some primer pairs to amplify genomic DNA templates in our study.

All primer pairs that were originally derived from *P. x hortorum* DNA were functional in most *P. peltatum* accessions and *vice versa*, consistent with the conserved nature of microsatellite loci in other plant genera (e.g. in *Actinidia*; Weising et al. 1996; Huang et al. 1998). Nevertheless, group-specific, 'diagnostic', alleles were not uncommon, especially for the diploid *P. peltatum* cultivars. Four primer pairs (PhI28, PhI39, PhI45, PhI63) cloned from *P. x hortorum* failed to amplify several of the *P. peltatum* varieties (particularly the diploids), while 2 primer pairs (PpG13 and PpG14b) cloned from *P. peltatum* did not produce amplification products for the wild variety *P. p. ssp. lateripes*, and DRE, a diploid accession believed to be closely related to *P. p. ssp. lateripes*. As repeated experiments gave consistent results, these non-amplifiable loci were classified as 'null alleles'. Null alleles are common in cross-species amplifi-

cations and are presumably due to mutations in the primer binding sites (Callen et al. 1993; Koorey et al. 1993).

Based on the history of cultivation, Renou et al. (1995) suggested that the genetic base of *Pelargonium* varieties may be narrow. This hypothesis was corroborated by PCR with arbitrary primers (Renou et al. 1995; Starman and Abbitt 1997; Lesur et al. 1999) and could explain the relatively low degree of polymorphism we found across the *Pelargonium* varieties. The extent of microsatellite polymorphism in other cultivated plant species ranges from 1–16 alleles in bread wheat (*Triticum aestivum*; Bryan et al. 1997; Plaschke et al. 1995), at the less polymorphic end of the spectrum, to 3–36 alleles in the highly polymorphic kiwifruit (*Actinidia chinensis*; Weising et al. 1996; Huang et al. 1998). In *Pelargonium*, the most polymorphic locus out of the 18 tested had 11 alleles, but most loci had fewer than 10, yielding an average of only 6.5 alleles per locus.

Notwithstanding the comparatively low average polymorphism, 41 out of the 44 accessions examined could be uniquely described by allelic variation at just 3 loci (PhI21, PhI45, PhI49). The probabilities of obtaining a particular phenotype using data from these loci only ranged from as low as 9.91×10^{-9} (REN; Table 1) to approximately 0.001 (WES; Table 1). These probabilities are likely to be sufficient to characterise varieties currently registered as protected. Moreover, the probabilities can be substantially improved by adding data from 1 or more of the remaining 26 loci. Of the cultivars that could not be distinguished, 2 were known somatic mutants of a third ('Roi des Balcons'). To our knowledge, the remaining cultivar, Evka, is not a part of this sport family. However, like the Roi des Balcons accessions, Evka is a diploid member of the *P. peltatum* group with a phenotypic aspect quite similar to that of the Roi des Balcons family. The possibility that this variety is a cryptic member of the Roi des Balcons group can therefore not be excluded. However, it is also possible that the high number of null alleles in diploid *P. peltatum* accessions obscures the true relationship between these cultivars. Cloning of additional microsatellite loci from a diploid *P. peltatum* library should improve the resolution of this group.

The high discriminatory power of the microsatellite loci examined in this study compares favourably with that reported for microsatellites used in cultivar identification of other species. For example, 2 out of 40 wheat cultivars could not be distinguished by 23 wheat microsatellite loci (Plaschke et al. 1995). In tomato, primer pairs specific for 4 microsatellite loci were needed to distinguish between 16 inbred cultivars and F₁ hybrids (Bredemeijer et al. 1998). Several studies have focussed on variety identification in apple. Guilford et al. (1997) found 3 microsatellite markers to be sufficient to discriminate all 21 cultivars examined, whereas 2 markers separated 18 out of 19 cultivars in a study by Gianfranceschi et al. (1998). Hokanson et al. (1998) were able to distinguish all but 8 pairs of a core collection of 66 apple accessions with 8 primer pairs. In the latter two studies, most of the indistinguishable genotype pairs were sport mutations and/or

their progenitors. Flower colour sports could also not be distinguished in our study. Since flower colour variation is presumably due to point mutations that do not affect all three germ layers, it is highly unlikely that microsatellite markers would detect such mutations. Considering their otherwise high discriminatory power, microsatellites will hence be useful to assign 'essentially derived varieties' such as sports and transgenics to their parental origin.

In our UPGMA analysis, tetra- and diploid *P. x hortorum* accessions formed two very closely related groups, whereas tetraploid and diploid *P. peltatum* plants fell into two very distinct assemblages. Similar groupings were obtained by earlier RAPD studies (Renou et al. 1995; Lesur et al. 1999). However, possibly due to the 'dominant' character of RAPD markers, diploid and tetraploid *P. x hortorum* accessions could not be distinguished. Although the determination of exact tetraploid genotypes is not always possible, the co-dominant inheritance of microsatellites nevertheless gives a more accurate description of allelic diversity, which permitted us to distinguish di- and tetra-ploid accessions.

The low level of diversity within *P. x hortorum* compared with *P. peltatum* is surprising. *P. x hortorum* varieties are believed to result from numerous interspecific hybridisations, involving mainly *P. zonale* and *P. inquinans*, but also *P. salmoneum* and *P. scandes*. In contrast, *P. peltatum* is considered to be a pure species. As has already been hypothesised by Renou et al. (1995), the relative heterogeneity between diploid and tetraploid *P. peltatum* varieties suggests either that these were separated very early in the breeding history or, alternatively, that tetraploids arose from allopolyploidisation events. The fact that *P. x hortorum* accessions typically possessed only a subset of the generally greater number of alleles observed in *P. peltatum* plants may indicate an early hybridisation event between the two groups. The low level of polymorphism in *P. x hortorum* accessions may reflect the selection pressure exerted by breeders attempting to stabilise characters such as floribundity, colour or plant growth. Extension of the present study to natural varieties will permit verification of this hypothesis.

Set 1 (Table 1) of the cultivars examined for this study was believed to be representative of pelargoniums available on the European market. The scoring of microsatellite loci of a further 21 accessions (Set 2, Table 1) did not reveal additional microsatellite alleles (but several additional allelic phenotypes), indicating that the varieties selected were indeed representative. The fact that only 3 of our 29 polymorphic microsatellite loci were sufficient to distinguish among all selected varieties suggests that cultivar identification, whether as a means to control illegal propagation or as part of the DUS test, is likely to be greatly assisted by the use of microsatellite markers. Marker-assisted selection is a distinct possibility as a future development in *Pelargonium* breeding, and steps to produce a skeleton map of the *Pelargonium* genome are currently under way.

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