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Microsatellite DNA in *Actinidia chinensis*: isolation, characterisation, and homology in related species

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Abstract We have isolated and sequenced 263 microsatellite-containing clones from two small insert libraries of *Actinidia chinensis* enriched for (AC/GT) and (AG/CT) repeats, respectively. Primer pairs were designed for 203 microsatellite loci and successfully amplified from both plasmid and *A. chinensis* genomic DNA. In this paper we report the sequences of 40 primer pairs for which we have demonstrated Mendelian segregation in the progeny from controlled crosses. The polymorphism of ten microsatellites of each type was evaluated in four diploid and six tetraploid genotypes of *A. chinensis*. All microsatellites proved to be polymorphic, the number of alleles per locus detected in polyacrylamide sequencing gels ranging from 9 to 17. The high degree of polymorphism in *Actinidia* renders these markers useful either for mapping in *A. chinensis* or for fingerprinting cultivars of both domesticated kiwifruit species (*A. chinensis* and *A. deliciosa*). While most primer pairs produced single amplification products, about 20% generated banding patterns consistent with the amplification of two different loci. This supports the hypothesis that diploid species of *Actinidia* ($2n = 2x = 58$) are polyploid in origin with a basic chromosome number $x = 14/15$ and that chromosome duplication may have occurred during the evolution of the genus. Finally, we have assayed the cross-species transportability of primer pairs designed from *A. chinensis* sequences and have found extensive cross-species amplification within the genus *Actinidia*; 75% of primer pairs gave successful amplification in the eight species assayed (*A. arguta*, *A. rufa*, *A. polygama*, *A. chrysantha*, *A. callosa*, *A. hemsleyana*, *A. eriantha*, and *A. deliciosa*), which are representative of the four sections into which the genus is currently split.

Key words Simple sequence repeat (SSR) · Microsatellites · Molecular markers · Genetics · Kiwifruit

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Introduction

Microsatellites, also known as simple sequence repeats (SSRs), are short (1–5 bp long) tandemly repeated, DNA sequences, (Tautz 1989). They occur frequently throughout eukaryotic genomes (Morgante and Olivieri 1993; Wang et al. 1994) and are highly polymorphic as a result of the frequent variation in the number of times the core sequence is repeated (Tautz 1989). They are inherited in a co-dominant Mendelian manner (Morgante and Olivieri 1993) and are easily detected by means of the polymerase chain reaction (PCR) and gel electrophoresis (Rafalski et al. 1996). All these positive features make microsatellites suitable markers for several applications in genetic analysis. They sometimes have conserved flanking regions, so that heterologous primer pairs can provide useful markers in related genomes (Moore et al. 1991), and are now replacing other molecular markers in the construction of linkage maps in human and other mammals (Beckmann and Soller 1990). Until recently, microsatellites have not been widely used in plant studies because of the high cost of their isolation and sequencing, but the introduction of procedures such as automatic sequencing and library enrichment, which rendered the process more efficient, now place these markers within the reach of plant geneticists (Gupta et al. 1996; Rafalski et al. 1996). Microsatellites have recently been isolated and characterised in several crops, including

fruit crops such as grape (Thomas and Scott 1993), kiwifruit (Weising et al. 1996), citrus (Kijas et al. 1995), apples (Guilford et al. 1997), avocados (Sharon et al. 1997) and peaches (Cipriani et al. 1997).

The kiwifruit [*Actinidia deliciosa* (A Chev) CF Liang and AR Ferguson] is a recently domesticated fruit crop, and its production in almost all parts of the world relies on a single female cultivar. Consequently, there is a demand for new kiwifruit cultivars (Ferguson et al. 1996). The genus *Actinidia* is genetically very diverse, consisting of more than 60 species (Liang 1984), some of which possess interesting traits, such as plant growth habit, fruit size and shape, skin colour, flesh quality and appearance. However, the introgression of such traits from wild germplasm into commercial cultivars requires many generations, and unfortunately *Actinidia*, like other woody fruit crops, has a long juvenile period (Testolin et al. 1995). Breeding programs can be accelerated by the use of molecular markers, which facilitate the selection of desired characteristics on the basis of genotype rather than phenotype. Molecular markers also allow early selection of traits linked to a marker but not expressed during the juvenile phase, such as sex or any fruit trait.

With the aim of producing markers for mapping the *Actinidia* genome, we have initiated a systematic isolation and sequencing of microsatellites in diploid *A. chinensis* Planch., a species closely related to, and a probable progenitor of, the hexaploid *A. deliciosa* (Testolin and Ferguson 1997). We have isolated and characterised a substantial number of (AC/GT)*n* and (AG/CT)*n* microsatellites, have checked their inheritance in controlled crosses, have screened them for genetic polymorphism using a panel of diploid and tetraploid genotypes of *A. chinensis*, and have investigated their transportability to other *Actinidia* species.

Materials and methods

Plant material

Genomic libraries were constructed from diploid *A. chinensis* (accession #54.20) cultured in vitro. SSR polymorphism was assayed using a panel of four diploid genotypes of *A. chinensis* (#54.1, #54.20, #54.23, #54.19) and six tetraploid genotypes (#136, #137, #138, #144, #139, #C134.4). Segregation tests were carried using seven F1 offspring derived from either one of two families produced through controlled crosses (*A. chinensis* #54.23 × *A. chinensis* #54.19 and *A. chinensis* #54.20 × *A. callosa* #103). The particular family chosen depended on the polymorphism in the parents. The plants of different species used to assess the transportability of SSRs isolated from *A. chinensis* were: *A. arguta* (accession #61, tetraploid), *A. rufa* (#124.1, diploid), *A. polygama* (#69, diploid), *A. chrysantha* (#104.2, tetraploid), *A. callosa* (#103, diploid), *A. hemsleyana* (#50, diploid), *A. eriantha* (#105.1, diploid), and *A. deliciosa* cv "Hayward" (#46, hexaploid). These species are representative of the four sections into which the genus has been split (Liang 1984). The plants came from the living collection of *Actinidia* germplasm held at the Agriculture Experimental Station of the University of Udine, Italy.

DNA extraction

Total DNA was extracted from about 1.0 g of young leaves according to the procedure described by Doyle and Doyle (1990), as modified slightly by Cipriani and Morgante (1993), and purified with 2-butoxyethanol according to Manning (1991).

Construction of the genomic libraries enriched in SSRs

Genomic DNA from *A. chinensis* #54.20 was digested with *Tsp509I*. DNA fragments were separated on a 2% agarose gel and those in the size range of 200–800 bp were eluted onto a NA-45 DEAE membrane (Sleicher and Schuell). Fragment recovered from the membrane were ligated to synthetic adaptors, denatured and hybridised to biotinylated $(GT)_{13}$ or $(CT)_{13}$ oligonucleotides. Oligo-fragment hybrids were selectively separated from the remaining DNA using streptavidin-coated paramagnetic beads (Dynabeads, Dynal, Oslo, Norway). The DNA fragments containing the desired SSR repeats were eluted as single strands from the beads and the second strand was successively regenerated and amplified by PCR, using adaptor-specific primers (Rafalski et al. 1996), to produce two genomic libraries enriched either in (AC/GT)*n* or (AG/CT)*n* microsatellite repeats respectively.

DNA fragments recovered after the enrichment procedure were digested with *Eco*RI, cloned into the *Eco*RI site of *Lambda Zap* II vector (Stratagene) and the recombinant phages were used to transform XL1-Blue MRF′ *Escherichia coli* cells (Stratagene) using standard protocols.

Plaque screening for positive clones

Plaques plated at low density (< 100 colonies/100-mm plate) were lifted onto a Hybond-N nylon filter according to standard procedures (Sambrook et al. 1989) and screened by plaque hybridisation with a digoxigenin-labelled synthetic (AC/GT) polynucleotide (Pharmacia) probe. The probe was prepared according to the random-prime method using the DIG-High Prime protocols (Boehringer Mannheim). Filters were washed at 60°C in 1 SSC plus 0.1% SDS. Positive plaques were individually recovered. A second library, produced as described above, was enriched and screened for (AG/CT)*n* SSRs using a digoxigenin-labelled synthetic (AG/CT) polynucleotide (Pharmacia) probe and using the same stringency for washes.

Anchor PCR

In order to minimise the amount of unsuccessful sequencing, positive clones were pre-screened for insert length, repeat position, and orientation by means of anchor PCR (Rafalski et al. 1996), a technique based on five PCR reactions carried out using a combination of four different primers: two vector primers and two degenerated primers specific to the repeat. Clones in which the repeat was either too far from both cloning sites to be sequenced or too close to one end of the insert, so that there was not enough room to design a suitable primer, were discarded.

Plasmid excision and sequencing

The pBluescript plasmid was excised in vivo from clones selected by the anchor PCR using the Stratagene protocol and used to transform SOLR *E. coli* competent cells (Stratagene). Colonies plated at low density were grown overnight on LB broth enriched with ampicillin and then recovered. The plasmid DNA was purified with

a Quantum Prep Plasmid Miniprep kit (BioRad) following the instruction manual.

The insert contained in the plasmid was sequenced with an A.L.F. Pharmacia Automatic Sequencer using a Thermo sequenase-cycle sequencing kit (Amersham) and 200 ng of plasmid DNA as a template.

PCR amplification and product electrophoresis

Sequences were first compared to the data base of previously sequenced SSRs using the FastA software in the GCG (Genetic Computer Group) package (University of Wisconsin, USA) to identify and remove duplicates; then PCR primer pairs were designed in the microsatellite flanking regions using Primer Software Package version 0.5 (Lincoln and co-workers, Whitehead Institute of Biochemical Research, Cambridge Mass. USA).

The pairs of primers were designed to be 18–22 bp long with an annealing temperature between 55 and 63°C (optimum 60°C) and an expected product length with few exceptions, in the range of 100–200 bp and then assayed in both plasmid and the original *A. chinensis*, as well as in the parents used for the controlled crosses.

PCR reactions were performed in a 25-µl vol containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 100 ng of genomic DNA and 0.2 U of *Taq* polymerase (Pharmacia) using the following temperature profile: 95°C for 5 min, then 35 cycles of 94°C for 50 s, followed by 55°C for 50 s and 72°C for 50 s, finishing with 72°C for 8 min. PCR products were separated by electrophoresis in 3% Metaphor (FMC BioProducts) agarose gels and stained with ethidium bromide.

Segregation analysis, polymorphism evaluation and fragment sizing in *A. chinensis*

Twenty microsatellites of each type, which were apparently heterozygous in at least one of the parents used in the two controlled crosses, were assayed for the segregation analysis. PCR amplifications and electrophoreses were carried out as described above.

Ten microsatellites of each type were assayed for their polymorphism in the panel of *A. chinensis* genotypes (see above). PCR reactions were carried out as described above, except that one of the two primers for each pair was radioactively labelled using γ-^{33}P-ATP and polynucleotide kinase (Sambrook et al. 1989). The labelled PCR products were separated on 6% denaturing polyacrylamide gels (Long Ranger™, FMC BioProducts) containing 7 M urea and run with a 1.2 × TBE buffer at a constant power of 55 W. The gels were then dried and autoradiographed on X-ray film using standard procedures. A reference molecular-size marker was prepared from a pBluescript plasmid (Stratagene) containing an *A. chinensis* genomic DNA insert of known sequence, which was sequenced using the M13 sequencing primer (Pharmacia), radioactively-labelled as above, and the four sequencing reactions loaded onto the gel.

Assay of microsatellites in fragments amplified from different *Actinidia* species

All primers were used to amplify the DNA of the different species. Two microliters of the PCR products were spotted on a Hybond-N nylon filter and hybridised at 60°C overnight to a poly(AG/CT) or poly(AC/GT) digoxigenin-labelled probe according to the procedures described above. In two cases where dot-blotting gave weak signals in several species, the DNA was sequenced in two species sampled among those giving a strong and a weak signal respectively. The PCR products were cloned using the pCR-Script Cloning Kit (Stratagene) following the manufacturer's instruction and sequenced as previously described.

Results

Efficiency of microsatellite identification procedures

As a result of the enrichment procedure about 50% of the plaques contained the target repeat. The anchor PCR test suggested that about 25% of the clones recovered from positive plaques should be discarded as being unsuitable for sequencing because the repeat was either too close to, or too far from, the cloning site. The remaining 276 clones were sequenced and of these 263 (95%) contained the insert in the output sequence (Table 1). No insert was found duplicated in the sequence data base.

Primer pairs were successfully designed for 203 sequences using Primer Software (Whitehead Institute, Cambridge Mass.) and the remaining 60 sequences were abandoned as they did not match the program parameters.

All sequences, for which primers were synthesised, were successfully amplified by PCR from both the plasmid and the *A. chinensis* genomic DNA template (Table 1) and the bands produced were of the expected size, indicating that the library contained negligible numbers of multiple inserts.

Microsatellite characterisation

Nearly all (95%) of both (AC/GT)*n* and (AG/CT)*n* microsatellite sequences were perfect with no interruption to the core-motif repeat sequence (Table 2). Almost half (45%) of the (AC/GT)*n* sequences were

Table 1 Efficiency of the procedure adopted for the identification of microsatellite markers in *A. chinensis*

Procedure step	(AC/GT)*n*		(AG/CT)*n*	
	n	%[a]	*n*	%[a]
Clones retained for sequencing after anchor PCR	191	–	85	–
Clones containing a microsatellite insert	180	94	83	98
Sequences suitable for primer design	139	73	64	75
Sequences correctly amplified in *A. chinensis*	139	73	64	75

[a] Calculated over the number of clones retained after anchor PCR

Table 2 Primer sequences, repeat motif, and PCR product sizes of 40 microsatellites sequenced from two DNA libraries of *A. chinensis* enriched with (AC/GT) and (AG/CT) repeats respectively

Locus code	Primer sequences (5′ → 3′)	Repeat motif	Length (bp)[a]
From the (AC/GT)*n* enriched library			
UDK96-001	5′ GAATCGCGTAATGATTGATGG 5′ GTTTCCCACTCTGCAAAAGC	(AC)10(TC)16	258
UDK96-009	5′ CACTCACATGCCTTTACACACA 5′ AAGAGGCCACCAAAAACCTT	(AC)8	172
UDK96-013	5′ ACGTGACTTGGTTTTTGAAGG 5′ CACTCCGATCAGCTCTCCTC	(AC)15	132
UDK96-015	5′ CCGAGTCATGATCGAGTTGA 5′ GGCTCAACTTGGAGAAGTGG	(AC)21	137
UDK96-016	5′ TTAGGTGAAAGACACACCACAC 5′ ATAAACGTCATGGGCTCAGC	(AC)14	199
UDK96-017	5′ CCCGTGAGATGTAACGTCAC 5′ TGAGAGAGAGAGTGGAGCTCG	(AC)21	162
UDK96-018	5′ GTAAACTGCATTTGGTCCTCG 5′ TTAGTTTTCATCCTTGGTTCCA	(AC)14	164
UDK96-019	5′ ATACACTTGAAGCGCCGC 5′ AAGCAGCCATGTCGATACG	(AG)11(AC)12	149
UDK96-020	5′ TTGGCTCGATTAACTGTTTA 5′ TCTGCTGTCAAACTGGATCA	(AC)15	92
UDK96-022	5′ GGCGTCAAGGTTCTCTCTCT 5′ TTGGGGTAACTACTCACTAAGC	(TC)9(AC)9	85
UDK96-023	5′ CCAAACGAGTCCTAGATCTTGC 5′ ATCTGGAGCCATACGGAGG	(TG)5(AC)14	145
UDK96-026	5′ CGCTGACCAGATTCTGATGA 5′ TTGAAAATCACTGAGCACAACC	(AC)14	173
UDK96-028	5′ TCCCCACACAACAACTCCTC 5′ CAGATGACCTGCACGTGC	(AG)19	150
UDK96-030	5′ TCATGTTTGTGGTTGAGTTGTG 5′ AGCAATAAACTCAAGCGCGT	(AG)9(AC)15	115
UDK96-033	5′ AGGCAGCAATGCTCTCTCTC 5′ CTGGTGGACAGGGCTAAAG	(AG)12(AC)15	130
UDK96-034	5′ TTATATGGTGCGGCATGCTA 5′ TGAATGCAGAAGGCAATCAG	(AC)12(AG)17	201
UDK96-035	5′ AAGAGCCATAGCTTATTCACCG 5′ AAGTAAAGCCATTGTCATTGCA	(AC)17	148
UDK96-037	5′ CATTTCAGTGTTAACTCTTTCG 5′ CCCTCTTTCAAAGGTTCTCT	(TG)10GC(TG)6(AG)20	122
UDK96-039	5′ GGTTTGATCGGTCTTCGAAA 5′ ATAAATGTGTGCCAGTGCGA	(AC)10	148
UDK96-040	5′ TCGAGTTACCTAGCTACTCCGC 5′ CAAGGGAAGAAAATGTTGAACC	(AC)15(AG)21	177
From the (AG/CT)*n* enriched library			
UDK97-401	5′ GAGCAAAAAGCTTGACACCC 5′ ACCTGGTGGTAGATCCTCCA	(AG)24	146
UDK97-402	5′ ATATGATAGAACGCCATCCCC 5′ GACGTGCCATCAGTTTCCTT	(AG)13AA(AG)19	126
UDK97-403	5′ CTTTTTTGAATTCGTCCCCA 5′ GCGGCGAAGGTGAATAATCA	(AG)15	150
UDK97-404	5′ CGGCATTTTCTTTTTAATGACC 5′ TTGCCTTGCTCTTGTTCATG	(TC)20	151
UDK97-405	5′ TACACGGAGTACAAAGGTGGG 5′ AACCGATGATCAAGAAAACCC	(AG)21	149
UDK97-406	5′ TTGACTTTTGGAAATCAAAACA 5′ CTCATACCTTTTGTGGATGTGG	(AG)22	120
UDK97-407	5′ CGTTCGAGGGTAAGGAGTTG 5′ CCTAACCCGACCGTAGTGTG	(AG)21	110
UDK97-408	5′ GTGCTCCTCCGTCCATGTAT 5′ CGTCCTCTCTTCGCCATTTA	(AG)21	111
UDK97-409	5′ ATGACCTATTGCCAAGTGGC 5′ TTGTGTGTACCCACCACCC	(AG)19	130
UDK97-411	5′ TGATTAAATCCCCAAACCCA 5′ TCCCAATCTCATCATCCCTC	(AG)21	148
UDK97-412	5′ TCAAACTCGAACCACATTAACA 5′ TAGCCTGTTGCCGGCTAG	(AG)20	100

Table 2 Continued

Locus code	Primer sequences (5′ → 3′)	Repeat motif	Length (bp)[a]
UDK97-413	5′ GCACTTTACACCTCTCAATCAA 5′ AAAGATGTTTTTGAACCATGCA	(AG)21	133
UDK97-414	5′ GCCATTTCAAGAACATTTTTTG 5′ TGGCTATATTTTGCAAGCCC	(AG)20	139
UDK97-415	5′ TCTTCAAATGGGCATGTTGA 5′ CCAGCCAATCTCTCCCAATA	(AG)21	145
UDK97-416	5′ GATTCTAGATTCGAGGCTCTGG 5′ AGAGCCCCTACTTATTGCTGC	(AG)20	137
UDK97-419	5′ TGGGTAGTTTCTCTGGTTTTGC 5′ CATGGGGTTTTCTAAAGACTGC	(AG)26	131
UDK97-420	5′ GAGCGTAGAATGTGGCGG 5′ CATTTGAAATGAGTCAAGCTGC	(AG)17	136
UDK97-421	5′ AATCGGACGTCGCTTCTG 5′ TCGTTGGGTCTCCAAATCTC	(AG)20	117
UDK97-422	5′ GGAGAGGTGATTGTGGCAGT 5′ CTTATCATCTGCGCCCTCTC	(AG)15	149
UDK97-424	5′ TCATATGACAACCACAAGCTCA 5′ TGATGTGCCTCTATCATGTGC	(AG)19	111

[a] Calculated from the plasmid sequence within the designed primer pair

compound, according to the terminology adopted by Weber (1990) in humans and extended to plants by Morgante and Olivieri (1993), with a second and in one case a third repeat stretch running adjacent to, or very close to, the main repeat. With one exception, these extra repeats were all of the (AG/CT)*n* type: the single exception was a repeat of the complementary sequence. All but one of the microsatellites sequenced from the (AG/CT)*n* enriched library were simple (Table 2). The two types of microsatellite sequences differed significantly in length. The (AC/GT)n sequences were short, on average $n = 14$ (range 8–21), whereas for the (AG/CT)*n* sequences $n = 20$ on average (range 15–26).

Allele segregation and length polymorphism of microsatellites in *A. chinensis*

Analysis of crosses involving only diploid genotypes indicated that the segregation of PCR products amplified in the parents was consistent with Mendelian inheritance of heterozygous loci except for a few cases in which the offspring of apparently heterozygous parents showed no segregation. This was probably due to two loci being present, and the analysis of the polymorphism carried out subsequently in several different genotypes confirmed that two different loci were indeed amplified in all cases. When segregation did occur, not all expected phenotypes appeared in the progeny. This sometimes happened when the parents were heterozygous for several different alleles and is probably due to the small number of progeny examined. An example of the segregation patterns is shown in Fig. 1.

The polymorphism of 20 microsatellites, ten of each type, was evaluated in four diploid and six tetraploid genotypes from different accessions of *A. chinensis*

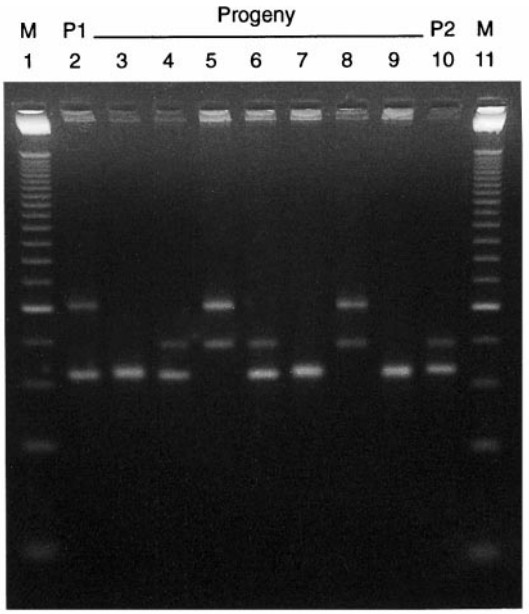

Fig. 1 Allele segregation of the microsatellite locus UDK96-037, sequenced in *A. chinensis*, in progeny from the cross *A. chinensis* #54.20 (P1) × *A. callosa* #103 (P2). *M* = 25-bp ladder

using standard polyacrylamide sequencing gels. The 'stuttered' ladder patterns commonly reported in the literature (Litt et al. 1993; Rafalski et al. 1996) were almost unique to each microsatellite as far as the total number of bands per allele and the number of bases separating one band from the next in the ladder were concerned (Fig. 2). Although it was difficult to determine which band represented the true allele, alleles could be easily distinguished from one another at a resolution of 2, and sometimes 1, bp.

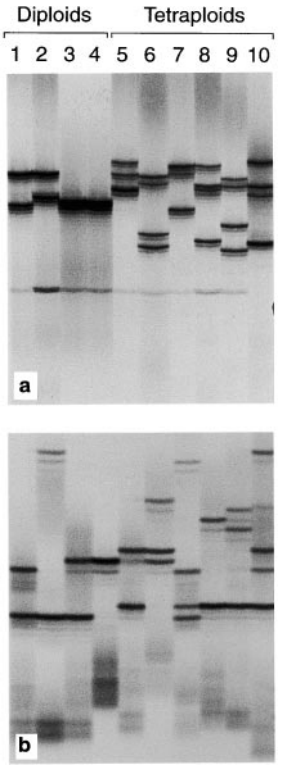

Fig. 2 Autoradiogram of a polyacrylamide-gel separation of PCR amplification products of ^{33}P-labeled UDK96-001 (**a**) and UDK96-015 (**b**) primer pairs from four diploid and six tetraploid genotypes of *A. chinensis*. The genotypes are those of Table 3. Note the different 'stuttering' patterns of the two loci

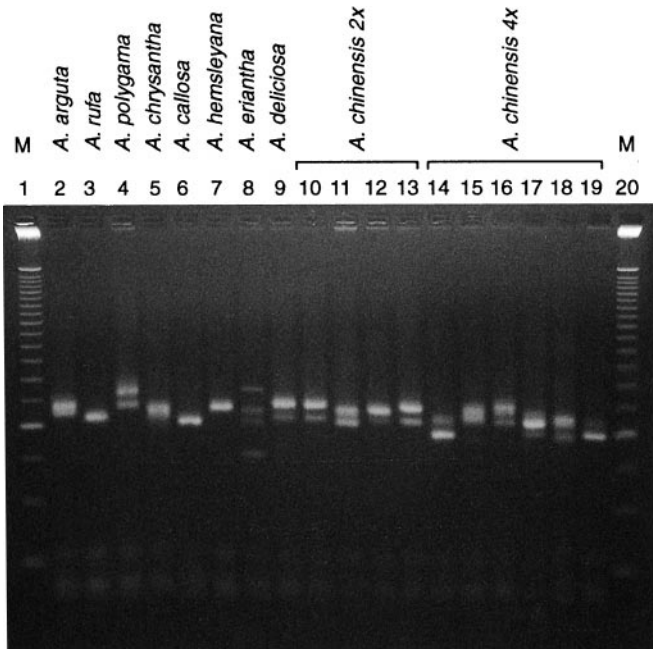

Fig. 3 PCR amplification and electrophoresis, on a 3% Metaphor agarose gel, of the microsatellite UDK96-033 in different species and genotypes of *Actinidia*

All microsatellites were highly polymorphic. For each primer pair, 9–17 different fragments were detected, though the number of alleles might have been underestimated as PCR amplification failed in one case and gave poorly staining bands in five cases for the genotype #134.4 (Table 3).

Of the 20 primer pairs assayed in the polyacrylamide-gel system, four (20%) detected more than one locus since the number of alleles was higher than would be expected for the ploidy of the genotype studied. In most cases, alleles belonging to different loci could be easily distinguished by analysis of segregation in the progeny, the different polymorphic banding patters observed, and, occasionally, the different patterns of shadow bands.

Microsatellite homology in different *Actinidia* species

PCR amplification of homologous microsatellites in different species was considered to be successful when sharp bands in the expected size range were obtained on agarose gels (Fig. 3). Using this criterion, 75% of microsatellites where apparently amplified in the eight *Actinidia* species surveyed (Table 4).

All 40 primer pairs gave successful amplification in *A. arguta* and *A. deliciosa*, 38/40 in *A. hemsleyana* and *A. rufa*, 37/40 in *A. eriantha*, 36/40 in *A. callosa* and *A. chrysantha* and 35/40 in *A. polygama*. Failure of amplification was usually detected simply by the absence of any PCR product, but in a few cases multiple bands and/or bands outside of the expected size range were obtained.

Whether or not amplification was considered to be successful, all PCR products, were hybridised with digoxigenin-labelled specific repeat probes. As expected, those PCR reactions that failed to amplify also gave no signal on dot blotting, except for two cases for which we have no simple explanation. Of the 342 positive PCR amplifications, seven (2%) gave no signal on dot blotting and we have concluded that these loci lacked the microsatellite repeat; the remaining 335 amplifications gave spots of variable intensity. These spots are taken to indicate that the microsatellite was present in the sequence. We sequenced the PCR products obtained in two species by two primer pairs (UDK96–015 and UDK96–018), which yielded weak and strong spots respectively on blotting, and compared the sequences with those of *A. chinensis*. In all cases the microsatellite repeat was found to be present, although the number of repeats varied considerably, the weaker spots corresponding to shorter repeat sequences. The multiple alignment of sequences generated by cross-species amplification of the UDK96–018 locus is reported in Fig. 4.

Table 3 Polymorphism of 20 microsatellite loci in four diploid and six tetraploid *A. chinensis* genotypes

Locus	Diploid genotypes				Tetraploid genotypes						No. of alleles
	#23.1	#23.20	#23.23	#23.19	#136	#137	#138	#144	#139	#134.4	
UDK96-001	275/261	275/265	263/261	263/261	281/275/267	275/271/247/241	279/259	279/269/243	273/269/251/239	281/269/265/241	17
UDK96-015[a]	113/105	137/105	115/105	115	117/107	127/117/115	135/113/107/105	123/107	125/121/107	137/117/107	11
UDK96-020	96/92	96/92	96	96	92/90	76/72	76/74	86	90/86	96/88/80	9
UDK96-022	97/95	97/87	97/95	97	99/93/87/85	97/85	97/91/89/87	95/91/87	105/93/85/83	95/83/80	11
UDK96-026[a]	161/147	181/147	161/147	151/147	163/143	167/161/157/145	161/157/145	159/145	161/147	189/179/173/141	14
UDK96-030	139/115	143/115	139/107	115/107	121/119/116/110	123	117/109/106	121/115/113	115/114/111	99/85	17
UDK96-033	138	134/122	134	138	132/116	138/134/130/126	142/128	130/126/118	132/130/116/112	118[b]	11
UDK96-034	205/189	201/189	205	205/189	203/195	231/181	195/191/187	193/183	213/195/191/181	?[b]	12
UDK96-037	79	126/81	79	130/79	85/81/77/75	89/77	89/85/77	92/83/81/75	91/87/83/77	116/104/102	15
UDK96-040	145/135	183/145	145/135	137/135	157/151/147/137	179/173/155/147	171/169/167/137	181/165/159/147	157/137	?[b]	16
UDK97-401[a]	130/112 (116)	152 (116)	152/112 (116)	152/126 (116)	132/128/118/112 (116)	142/130/122/112 (116)	130/128/126 (116)	138/126/124 (116)	134/130/126/124 (116)	112[b] (116)	12
UDK97-402	125	125/81	125/99	125	81	91/89/83/77	93/87/79/77	79/77	87/83/81/77	86/82/80/78	14
UDK97-403	152/150	150/146	152/142	152	158/152/150	164/144	152/146/132	154/144/132	152/144	160/154/136	11
UDK97-404	151	151	151/143	151/141	149/145/141/135	151/149/147	147/143/137	135/133	141/133	149/147/145/131	10
UDK97-405	147/135	151/145	145	145	117	153/125/117	147/117	135/133/127/123	135/117	–[c]	10
UDK97-406	107	117	107/95	95	117/109/103	109/103/89	115/113/105/101	121/107/97/93	119/107/105/91	91	15
UDK97-407	82	110/82	82	82	94/90/82	100/92/84	92/86	108/98/96/90	96/94	94/84	11
UDK97-408[a]	107/84 (102/100)	119/107 (100)	119/107 (102)	107 (100)	113/107/86/84 (102)	105/84 (101)	121/115/86/82 (102)	84 (102)	106/84/82 (102)	94/92/86 (?[c])	13
UDK97-409	120	130/118	112/104	120/104	130/118	128/122/120	124/120	118/112	128/122	118/114/112	9
UDK97-411	117	151/145	151/117	151/117	125/121/117	133/125	125/121/119	121/119/109	145/125/119	123/?[b]	10

[a] A second locus was amplified. Bands, when well scorable, are reported between brackets
[b] Weak amplification
[c] No amplification

Table 4 Transportability of 40 primer pairs, designed on DNA microsatellite flanking regions sequenced in *A. chinensis*, to different species of *Actinidia*

Locus code	Species of *Actinidia* successfully amplified[a]
UDK96-001	ALL but Pol, Hem, Eri
UDK96-009	ALL
UDK96-013	ALL
UDK96-015	ALL
UDK96-016	ALL but Eri
UDK96-017	ALL
UDK96-018	ALL
UDK96-019	ALL
UDK96-020	ALL
UDK96-022	ALL
UDK96-023	ALL but Pol
UDK96-026	ALL
UDK96-028	ALL
UDK96-030	ALL
UDK96-033	ALL
UDK96-034	ALL but Cal
UDK96-035	ALL
UDK96-037	ALL
UDK96-039	ALL but Pol, Cal
UDK96-040	ALL
UDK97-401	ALL
UDK97-402	ALL
UDK97-403	ALL
UDK97-404	ALL
UDK97-405	ALL
UDK97-406	ALL
UDK97-407	ALL but Chry, Eri
UDK97-408	ALL
UDK97-409	ALL
UDK97-411	ALL
UDK97-412	ALL but Ruf, Pol, Chry, Cal, Hem
UDK97-413	ALL
UDK97-414	ALL
UDK97-415	ALL but Chry
UDK97-416	ALL but Ruf, Pol, Chry
UDK97-419	ALL but Cal
UDK97-420	ALL
UDK97-421	ALL
UDK97-422	ALL
UDK97-424	ALL

[a] ALL = all species (see Materials and methods for the list of species assayed), Cal = *A. callosa*, Chry = *A. chrysantha*, Eri = *A. eriantha*, Hem = *A. hemsleyana*, Pol = *A. polygama*, Ruf = *A. rufa*

Discussion

The enrichment procedure allowed us to produce libraries in which most clones contained a microsatellite. This is a much higher proportion than was found in non-enriched libraries of *Actinidia* (Weising et al. 1996) or of other plants in which the percentage of positive clones seldom reached 1% of the total number of colonies.

Microsatellites sequenced from the (AC/GT)*n* library contained relatively short (AC/GT) repeats, a further repeat of the type (AG/CT) in 40% of cases and a different type in 5% of cases, whereas microsatellites sequenced from the (AG/CT)*n* library were mostly simple and of the expected type. This is indirect evidence in support of the conclusion of Weising et al. (1996) that in *Actinidia* (AG/CT) repeats are more common than (AC/GT) repeats, as is also the case in other plant genomes (Morgante and Olivieri 1993; Wang et al. 1994). Surprisingly we did not find any (AT)*n* repeat associated with the target repeats, although AT is by far the most common type of microsatellite repeat in plants (Morgante and Olivieri 1993; Wang et al. 1994). This may simply reflect biased sampling of the *A. chinensis* genome or else may indicate a peculiarity of this species.

All 20 microsatellites examined in *A. chinensis* were polymorphic, with most having a very large number of alleles. The high degree of polymorphism was due mainly to variation in the length of the microsatellite region, but we cannot exclude the possibility that polymorphism is also due to insertion/deletion events in the flanking sequences, as has been observed in other plants (Peakall et al. 1998). This possibility is supported by the occasional presence of odd-number-sized fragments in an otherwise even-number-sized series or vice versa (see, for example, the loci UDK96-022, UDK96-030, and others) and by the wide variation in allele size, which in some loci extends considerably beyond the length of the sequenced microsatellite repeat (see, for example, the locus UDK96-026).

The high heterozygosity of microsatellites means that a good proportion of them will segregate in any controlled cross, thus making it possible to obtain a linkage map for kiwifruit in a short time. We have estimated that in the few families obtained by controlled crosses of diploid *A. chinensis* up to about 50% of the microsatellites so far assayed could be useful in producing a linkage map for a given cross. Furthermore, the large number of alleles for each microsatellite makes them suitable for cultivar fingerprinting. Polyploidy increases the resolving power of each microsatellite (Weising et al. 1996): the domesticated kiwifruit (*A. deliciosa*) is hexaploid and some new promising cultivars of *A. chinensis* are tetraploid.

Most primer pairs produced single amplification products, but 20% produced more bands than predicted from the ploidy of the genotype assayed. This implies that some loci are duplicated. Although this is usually not a problem either in mapping or fingerprinting, more stringent amplification could be attempted by designing new or longer primers from the original sequence. Apparent duplication of loci nevertheless raises the question as to whether diploid species of *Actinidia* are truly diploid. The basic chromosome number in *Actinidia* is unusually high ($x = 29$), and McNeilage and Considine (1989) speculated that the genus could have developed at a stabilised tetraploid level from ancestors with $x = 15$ and/or 14. Soejarto

Fig. 4 Multiple alignment of sequences generated by cross-species amplification of the UDK96-018 microsatellite. Note the presence of the (TG)*n* repeat (*in bold*) in all species

```
                  1                                                  50
A. chrysantha     gtaaactgca tttggtcctc gctatgagtt gagtggattt tttttattat
A. hemsleyana     gtaaactgca tttggtcctc gctatgagtt gagtgga.tt tttttattat
A. chinensis      gtaaactgca tttggtcctc gcnatgagtg gattttntta ttattattat

                  51                                                 100
A. chrysantha     tattatttat tta.......  .........t ttttgg(tgtg)2 (tgtgtgtgtg)
A. hemsleyana     tattatttat tta.......  .......... ttttgggggg (tgtgtgtgtg)
A. chinensis      ttatttatnt ttngngtgtg antn(tgtgtg) (tgtgtgtgtg) (tgtgtgtgtg)

                  101                                                150
A. chrysantha     (tgtg)gatggg gattaaatat gagttagact tggaaaacta actaagtgga
A. hemsleyana     (tgtg)gatggg gggtaaatat gagttagact tgg.aaacta actaagtgga
A. chinensis      (tgtg)gatggg gattaaatat gagttagact tgg.aaacta actaagtgga

                  151             168
A. chrysantha     accaaggatg aaaactaa
A. hemsleyana     accaaggatg aaaactaa
A. chinensis      accaaggatg aaaactaa
```

(1970) listed 14 species of *Saurauia*, a genus closely related to *Actinidia*, with $2n = 30$ and one individual with $2n = 45$, from which he concluded a probable basic number of $x = 15$. Our frequent amplification of two loci with single primer pairs in diploid species seems to support the hypothesis that diploid species of *Actinidia* are themselves polyploid in derivation. The chromosomal localisation of microsatellites which amplify more than one locus could be a means of identifying chromosome duplication events which may have occurred during the evolution of *Actinidia*.

The transportability of microsatellites from *A. chinensis* to other species of the genus was very high and complete in at least two species (*A. arguta* and *A. deliciosa*), confirming what was preliminarily reported by Weising et al. (1996) for the same genus. We used a single primer pair for each microsatellite sequence, and it is probable that designing primers for different regions of the sequence could result in an even wider cross-species amplification. This extensive cross-species amplificability of microsatellite loci is not surprising as it has been demonstrated for very divergent taxa in cetaceans (Schloetterer et al. 1991), rodents (Kondo et al. 1993), birds (Primmer et al. 1996), fishes (Rico et al. 1996) and, to a lesser extent, in species related to crops such as barley (Saghai-Maroof et al. 1994), *Brassica* (Szewc-McFadden et al. 1996), the Cucurbitaceae (Katzir et al. 1996), soybean (Peakall et al. 1998), grape (Thomas and Scott 1993), and *Citrus* (Kijias et al. 1995). Further examples are summarised in Peakall et al. (1998). Cross-species transportability of microsatellite primers makes such markers useful tools for evolutionary and taxonomic studies.

The transportability of microsatellites between species means that polymorphic markers identified in one species as being useful for genetic analysis could also be useful in related species. Successful cross-species amplification indicates only that flanking regions are highly conserved, since PCR-amplification of bands within the expected size range does not guarantee either the presence of the repeat or that the repeat is long enough to allow polymorphism, both of which are pre-requisites for marker usefulness. Thus Peakall et al. (1998) demonstrated that some microsatellite loci yielded amplification products that provided little or no information across the genus *Glycine*. These authors also showed by DNA sequencing that differences between alleles identified in different species are more complex than simple changes in repeat numbers, as interruptions to the sequence and, more noticeably, variation in the sequence and the length of flanking regions may occur. There is a disappointing tendency for microsatellite alleles to be shorter in species other than that from which they were first isolated (Ellegren et al. 1995). Ellegren et al. (1997) demonstrated, by the use of microsatellite loci sequenced in both cattle and sheep in cross amplifications, that this pattern of smaller allele size in non-source species held in both directions. This supports the hypothesis of a sampling bias, due to the tendency to select the longest microsatellites in the source species. Whatever the cause, this does reduce the proportion of microsatellite loci which can provide information for related species. For example, only 10% of more than 1000 microsatellite loci sequenced in soybeans provide useful markers for related legume crops, such as cowpeas, broadbeans, and lupins (Peakall et al. 1998). We are confident, however, that in an obligatorily outcrossing genus such as *Actinidia* with its high marker polymorphism, a higher percentage of microsatellite loci sequenced in *A. chinensis* will also be useful as markers for genetic studies in other *Actinidia* species.

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References

Beckmann JS, Soller M (1990) Toward a unified approach to genetic mapping of eukaryotes based on sequence-tagged microsatellite sites. Biotechnology 8 : 30–32

Cipriani G, Morgante M (1993) Evidence of chloroplast DNA variation in the genus *Actinidia* revealed by restriction analysis of PCR-amplified fragments. J Genet Breed 47 : 319–326

Cipriani G, Lot G, Peterlunger E, Testolin R (1997) Possibilità di impiego di DNA microsatellite sequenziato in pesco nel *fingerprinting* di specie diverse del genere *Prunus*. Proc Identificazione molecolare e garanzie genetiche nella propagazione vivaistica delle piante da frutto. Cesena, Italy, May 9 (in press)

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12 : 13–15

Ellegren H, Primmer CR, Sheldon BC (1995) Microsatellite evolution — directionality or bias. Nature Genet 11 : 360–362

Ellegren H, Moore S, Robinson N, Byrne K, Ward W, Sheldon BC (1997) Microsatellite evolution — a reciprocal study of repeat lengths at homologous loci in cattle and sheep. Mol Biol Evol 14 : 854–860

Ferguson AR, Seal AG, McNeilage MA, Fraser LG, Harvey CF, Beatson RA (1996) Kiwifruit In: Janick J, Moore JN (eds) Fruit breeding. vol II: vine and small fruits crops. Wiley and Sons, New York, pp 371–417.

Guilford P, Prakash S, Zhu JM, Rikkerink E, Gardiner S, Bassett H, Forster R (1997) Microsatellites in *Malus* × *domestica* (apple): abundance, polymorphism and cultivar identification. Theor Appl Genet 94 : 249–254

Gupta PK, Balyan IS, Sharma PC, Ramesh B (1996) Microsatellites in plants — a new class of molecular markers. Curr Sci 70 : 45–54

Katzir N, Danin-Poleg Y, Tzuri G, Karchi Z, Lavi U, Cregan PB (1996) Length polymorphism and homologies of microsatellites in several Cucurbitaceae species. Theor Appl Genet 93 : 1282–1290

Kijas JMH, Fowler JCS, Thomas MR (1995) An evaluation of sequence-tagged microsatellite-site markers for genetic analysis within *Citrus* and related species. Genome 38 : 349–355

Kondo Y, Mori M, Kuramoto T, Yamada J, Beckmann JS, Somon-Chazottes D, Montagutelli X, Guenet JL, Serikawa T (1993) DNA segments mapped by reciprocal use of microsatellite primers between mouse and rat . Mammal Genome 4 : 571–576

Liang CF (1984) *Actinidia* In: Feng KM (ed) Flora Reipublicae Popularis Sinicae. Science Press, Beijing, vol 49 (2), pp 196–208, 309–324.

Litt M, Hauge X, Sharma V (1993) Shadow bands seen when typing polymorphic dinucleotide repeats: some causes and cures. Bio-Techniques 15 : 280–284

Manning K (1991) Isolation of nucleic acids from plants by different solvent precipitation. Anal Biochem 195 : 45–50

McNeilage MA, Considine JA (1989) Chromosome studies in some *Actinidia* taxa and implications for breeding. NZ J Bot 27 : 71–81

Moore SS, Sargeant LL, King TJ, Mattick JS, Georges M, Hetzel DJS (1991) The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. Genomics 10 : 654–660

Morgante M, Olivieri AM (1993) PCR- amplified microsatellites as markers in plant genetics. Plant J 3 : 175–182

Peakall R, Gilmore S, Keys W, Morgante M, Rafalski A (1998) Cross-species amplification of soybean (*Glycine max*) simple-sequence-repeats (SSRs) within the genus and other legume genera. Mol Biol Evol (in press)

Primmer CR, Moller AP, Ellegren (1996) A wide-range survey of cross-species microsatellite amplification in birds. Mol Ecol 5 : 365–378

Rafalski JA, Vogel JM, Morgante M, Powell W, Andre C, Tingey SV (1996) Generating and using DNA markers in plants: In: Birren B, Lai E (eds) Non-mammalian genomic analysis. A practical guide. Academic Press, New York, pp 75–134

Rico C, Rico I, Hewitt G (1996) 470 million years of conservation of microsatellite loci among fish species. Proc Roy Soc Lond B 263 : 549–557

Saghai-Maroof MA, Biyashev RM, Yang GP, Zhang Q, Allard RW (1994) Extraordinarily polymorphic microsatellite DNA in barley — species diversity, chromosomal locations, and population dynamics. Proc Natl Acad Sci USA 91 : 5466–5470

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schloetterer C, Amos W, Tautz D (1991) Conservation of polymorphic simple sequence loci in Cetacean species. Nature 354 : 63–65

Sharon D, Cregan PB, Mhameed S, Kusharska M, Hillel J, Lahav E, Lavi U (1997) An integrated genetic linkage map of avocado. Theor Appl Genet 95 : 911–921

Soejarto DD (1970) *Saurauia* species and their chromosomes. Rhodora 72 : 81–93

Szewc-McFadden AK, Kresovich S, Bliek SM, Mitchell SE, McFerson JR (1996) Identification of polymorphic, conserved simple sequence repeats (SSRs) in cultivated *Brassica* species. Theor Appl Genet 93 : 534–538

Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res 17 : 6463–6471

Testolin R, Ferguson AR (1997) Isozyme polymorphism in the genus *Actinidia* and the origin of the kiwifruit genome. Systematic Bot 22 : 1–16

Testolin R, Cipriani G, Costa G (1995) Sex segregation ratio and gender expression in the genus *Actinidia*. Sex Plant Reprod 8 : 129–132

Thomas MR, Scott NS (1993) Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor Appl Genet 86 : 985–990

Wang Z, Weber JL, Zhong G, Tanksley SD (1994) Survey of plant short tandem DNA repeats. Theor Appl Genet 88 : 1–6

Weber JL (1990) Informativeness of human (dC-dA)*n*. (dG-dT)*n* polymorphisms. Genomics 7 : 524–530

Weising K, Fung RWM, Keeking DJ, Atkinson RG, Gardner R (1996) Characterization of microsatellites from *Actinidia chinensis*. Mol Breed 2 : 117–131