Theor Appl Genet (2005) 112: 149–157
DOI 10.1007/s00122-005-0117-x

ORIGINAL PAPER

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Cross-species amplification of microsatellite loci within the dioecious, polyploid genus *Actinidia* (Actinidiaceae)

Received: 13 July 2005 / Accepted: 14 September 2005 / Published online: 7 October 2005
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Abstract Microsatellite marker transfer across species in the dioecious genus *Actinidia* (kiwifruit) could offer an efficient and time-effective technique for use during trait transfer for vine and fruit improvement in breeding programmes. We evaluated the cross-species amplification of 20 EST-derived microsatellite markers that were fully informative in an *Actinidia chinensis* mapping family. We tested all 20 markers on 120 genotypes belonging to 21 species, 5 with varieties and/or chromosome races. These 26 taxa included 16 diploids, 7 tetraploids, 2 hexaploids and 1 octaploid, and represented all four taxonomic sections in the genus. All 20 markers showed some level of cross-species amplification. The most successful marker amplified in all genotypes from all species from all sections of the genus, the least successful amplified fragments only in *A. chinensis* and *A. deliciosa*. One species, *A. glaucophylla*, failed to amplify with all but 2 markers. PIC (Polymorphism information content) values were high, with 14 of 17 markers recording values of 0.90 and above. Sequence data demonstrated the presence of the microsatellite in all the amplified products. Sequence homology was less 5′ of the microsatellite and increased toward the start codon of the translated region of the EST from which the marker was derived. The data confirm that EST-derived microsatellite markers from *Actinidia* species show cross-species amplification with high levels of polymorphism which could make them useful markers in breeding programmes.

Communicated by I. Paran

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Introduction

Microsatellites or simple sequence repeats (SSRs) of two to five nucleotides in a tandem repeat pattern are recognised as powerful and informative genetic markers. They are abundant and spread throughout the genome, codominant, highly polymorphic, even within populations, and are easily amplified by polymerase chain reaction (PCR) (Byrne et al. 1996; Morgante and Olivieri 1993). The high level of polymorphism of microsatellite markers is the result of unusually high mutation rates for the nucleotide sequences (Peakall et al. 1998), and the variable number of unit repeats has been attributed to replication slippage, and possibly, unequal crossing-over events at meiosis (Schlotterer and Tautz 1992; Valdes et al. 1993). While a high level of heterozygosity could be expected in out-crossing species, highly inbred soybean cultivars were still found to have an average heterozygosity of 0.87 at seven microsatellite loci (Rongwen et al. 1995).

Genetic mapping in model plants such as *Arabidopsis* and rice has allowed comparisons with other species and has established that similarities of genomic structure among plant species exist (Gebhardt et al. 2003; Grant et al. 2000).

Cross-species transfer of microsatellite markers of genomic origin has now been demonstrated in many genera. Wheat SSR markers transferred to rye with 17% efficiency while 25% of rye markers were amplified in wheat. Markers from both wheat and rye were also transferable to triticale with 58% and 39% efficiency respectively (Kuleung et al. 2004). Peakall et al. (1998) demonstrated widespread cross-species amplification within *Glycine*, and some cross-species amplification among other genera. Transfer of SSR markers among crop species such as *Brassica*, vines (*Vitis, Actinidia*), fruit trees (*Citrus, Malus, Pyrus, Prunus*) and timber species (*Quercus, Pinus*) have all been reported (Westman and Kresovich 1998; Di Gaspero et al. 2000; Huang et al. 1998; Kijas et al. 1995; Pierantoni et al. 2004;

Wünsch and Hormaza 2002; Isagi and Suhandono 1997; González-Martínez et al. 2004).

As indicated by the results of Kuleung et al. (2004), transfer rates are variable among species. For example, 55–60% in *Glycine* (Hempel and Peakall 2003; Peakall et al. 1998), 17–100% in *Pinus* (Echt et al. 1999), 22–78% in *Quercus* (Isagi and Suhandono 1997), and in *Prunus* species (Cipriani et al. 1999) reported that 10 of 17 microsatellites (59%) gave apparently correct amplification across species.

It has been suggested (Cordeiro et al. 2001; Peakall et al. 1998; Scott 2001), that to increase transfer rates across all genetic distances, the microsatellites chosen as markers should be anchored within genes. It could be expected that transcribed regions would be more highly conserved, and microsatellite markers derived from ESTs (expressed sequence tags) would show correspondingly high cross-species amplification.

Cordeiro et al. (2001) report that, while highly transferable, EST-derived microsatellites tend to be less polymorphic within source and target species as there is selective pressure for sequence conservation in regulatory and functional genes. However, their greater homology across species compared with markers obtained from non-coding regions of the genome should give more accurate marker-trait associations, making such markers preferable for use in plant breeding programmes.

With the increase in EST databases associated with important crop species, microsatellite markers from ESTs are now a viable option in many genetic studies and applications. The microsatellites are required to be both transferable across species and polymorphic. In bread wheat 55% of EST-SSR markers exhibited transferability from *Triticum* to *Hordeum*, though a low level of polymorphism was detected compared to that seen with genomic SSRs (Gupta et al. 2003). Liewlaksaneeyanawin et al. (2004) reported a similar result for *Pinus* species. They also found that while EST-SSRs had high transfer rates across species when compared to genomic microsatellites, the level of polymorphism of the EST-SSRs was lower. These results would suggest that more EST-derived microsatellite markers may have to be developed to detect polymorphism, but such markers could be highly transferable across species and genera, and specific in marking traits.

The genus *Actinidia* is native to China and neighbouring countries and more than 70 species have been described. Within this number are *Actinidia deliciosa* (A.Chev.) C.F. Liang and A.R. Ferguson var. *deliciosa* ‘Hayward’, the green-fleshed kiwifruit, and *Actinidia chinensis* Planch. var. *chinensis* the yellow-fleshed kiwifruit ‘Hort16A’. Both these cultivars are marketed world-wide and are important economic crops in New Zealand. These two species are closely related and various breeding tools such as sex markers (Gill et al. 1998) have proved useful in both species dependent on the lineage of the population. There are, however, numerous other *Actinidia* species in which vine habit and fruit characteristics such as flesh colour, skin colour, change of colour on ripening, flavour components, or health attributes such as levels of vitamin C, are of interest to breeders.

All species of *Actinidia* are dioecious, and therefore out-breeding. In addition, the species form a polyploid series from diploid ($2x=58$), to octaploid ($8x=232$), and several species contain different ploidy races. *A. chinensis* has a haploid chromosome number of 29, but while this species is a functional diploid, experimental evidence would suggest that it is probably a palaeopolyploid (McNeilage and Considine 1989; Fraser et al. 2004; Huang et al. 1998).

To assist active breeding and genomics programmes in the two *Actinidia* horticultural species, a number of EST libraries have been generated from various tissues at different stages of development. From these libraries, microsatellites that are perfect dinucleotide repeats of more than 20 bases have been identified, and are being used to construct a genetic map in a selected family of *A. chinensis*. Both *A. chinensis* and *A. deliciosa* were source species for the microsatellites selected. When these markers were tested for mapping purposes in *A. chinensis*, a high level of polymorphism was found. The primers were designed from the transcribed region of a gene, and in most instances one primer of a pair was based on the translated sequence (Fraser et al. 2004).

In a preliminary study, a set of nine microsatellite markers developed from a genomic library of hexaploid *A. deliciosa* ‘Hayward’, was tested in three *Actinidia* species, representing three different ploidy levels. Of the nine markers which were polymorphic in an *A. deliciosa* family, eight were polymorphic in a family of diploid *A. chinensis*. In the more distantly related, tetraploid *A. arguta*, only four of the nine markers were informative (Fraser et al. 2001). These markers were derived from non-coding areas of the genome, but we expected a higher degree of transferability in the new set of EST-derived markers.

We have examined the cross-species amplification of a set of 20 EST-derived microsatellite markers with genotypes representing 21 species, and all levels of ploidy in *Actinidia*. We wished to evaluate the transferability of the markers across species, and the information content of such markers, to evaluate their usefulness in marker-assisted breeding and the mapping of agronomic traits.

Materials and methods

Plant material

A total of 120 genotypes from 27 taxa representing 21 species, and covering the four taxonomic sections of the genus *Actinidia* were tested (Table 1). All species were growing in the HortResearch orchards in Te Puke, Kerikeri and Riwaka, New Zealand. Young small leaves

Table 1 The *Actinidia* species tested, their Section, ploidy levels and the geographic location from which they originated

Taxon	Section	Ploidy	Geographic locations
A. chinensis Planch. var. *chinensis*	Stellatae	2*x*	New Zealand cultivar, Beijing, Guangxi, Henan, Jiangxi, Hunan, Shaanxi China
A. chinensis Planch. var. *chinensis*	Stellatae	4*x*	Jiangxi, Fujian China
A. arguta (Sieb. and Zucc.) Planch. ex Miq. var *arguta*	Leiocarpae	4*x*	Japan, Sakhalin, Korea, Beijing China, United Kingdom (ex China)
A. arguta var. *purpurea* (Rehd.) C.F. Liang (syn. *A. purpurea* Rehd.)	Leiocarpae	8*x*	United Kingdom (ex China), Sichuan China
A. callosa Lindl. var. *henryi* Maxim.	Maculatae	2*x*	United Kingdom (ex China), Sichuan China
A. callosa Lindl. var. *henryi* Maxim.	Maculatae	4*x*	Sichuan China
A. chrysantha Merr.	Maculatae	4*x*	Guangxi China
A. deliciosa (A. Chev.) C.F. Liang and A.R. Ferguson var. *deliciosa*	Stellatae	6*x*	Henan, Shaanxi, Guangxi, Hunan Sichuan, Yunnan, China
A. deliciosa var. *coloris* T.H. Lin and X.Y. Xiong	Stellatae	6*x*	Hunan China
A. fulvicoma Hance	Stellatae	2*x*	Zhejiang China
A. eriantha Benth.	Stellatae	2*x*	Fujian, Guangxi China
A. glaucophylla F. Chun var. *robusta*	Maculatae	2*x*	Guangxi China
A. guilinensis C.F. Liang	Stellatae	2*x*	Guangxi China
A. hemsleyana Dunn	Strigosae	2*x*	Fujian China
A. hypoleuca Nakai	Leiocarpae	2*x*	Japan
A. indochinensis Merr.	Maculatae	2*x*	Guangxi China
A. lanceolata Dunn	Stellatae	2*x*	Jiangxi China
A. latifolia (Gardn. and Champ.) Merr.	Stellatae	2*x*	Guangxi China
A. macrosperma C.F. Liang	Leiocarpae	4*x*	Guangdong, Beijing China
A. melanandra Franch.	Leiocarpae	2*x*	China
A. melanandra Franch.	Leiocarpae	4*x*	Beijing, Hilliers United Kingdom (ex China)
A. polygama (Sieb. and Zucc.) Maxim.	Leiocarpae	2*x*	Japan, Beijing China
A. rufa (Sieb. and Zucc.) Planch. ex Miq.	Maculatae	2*x*	Japan
A. setosa (Li) C. F. Liang and A.R. Ferguson	Stellatae	2*x*	France (exTaiwan)
A. valvata Dunn	Leiocarpae	4*x*	Jiangxi China
A. kolomikta (Maxim. and Rupr.) Maxim.	Leiocarpae	2*x*	Beijing China

Ploidy has been determined for the individual genotypes by flow cytometry reported in Ferguson et al. (1997), and personal communication with A.R. Ferguson

from 1 to 4 cm in length were collected shortly after budbreak, held at 4°C for 24 h, then stored at -80°C until required.

DNA extraction

DNA was extracted from leaf tissue of each genotype. Each sample was ground to powder in liquid nitrogen then processed through a DNeasy Plant Mini Kit (Qiagen™), according to the manufacturer's instructions. The volume of the eluate was 200 µl, and 5 µl of a one in ten dilution of each eluate was used in a PCR reaction.

PCR amplification and electrophoresis

A reaction mixture of 15 µl containing 1 x PCR buffer (20 mM Tris-HCl, 50 mM KCl), 5 mM $MgCl_2$ (the buffer and $MgCl_2$ were those supplied with the polymerase), 0.2 mM each of dNTPs, 4.5 pmol of each primer, and 1.25 units of Platinum Taq polymerase (Invitrogen), was prepared for each DNA sample. About 12.5 ng of genomic DNA was added in 5 µl to bring the total PCR volume to 20 µl. PCRs were performed in a Techne™ Genius thermal cycler with a single cycle of 94°C for 3 min preceding 35 cycles of denaturation at 94°C for 30 s, annealing at the optimum temperature for each pair of primers (at, or close to 60°C) for 30 s, and elongation at 72°C for 1 min. PCR reactions were carried out with primers labelled with 6FAM, VIC or NED (Applied Biosystems). The allelic content of each genotype was determined by capillary electrophoresis in an ABI Prism® 3100 Genetic Analyzer (Filter Set D, ROX™ size standard), and analysed with GeneMapper™ Software Version 3.0 (Applied Biosystems).

Polymorphism information content

Twenty fully informative markers as described in Fraser et al. (2004), which amplified one clear locus in the mapping family, were chosen to test across *Actinidia* species. Genetic diversity was measured by calculating the polymorphism information content (PIC). This calculation was made for diploid taxa, with 17 markers, the three excluded being those that amplified more than one locus in *A. chinensis*. In the presence of null and single-peak individuals, certain assumptions were made in order to estimate population parameters relating to allelic composition. We assume that all null alleles belong to one allelic category, and those individuals with a single peak are homologous. With these assumptions the PIC

was estimated from sample data using the formula $1-\sum_{i=1}^{n}p_i^2-2\sum_{i=1}^{n-1}\sum_{j=1}^{n}p_i^2p_j^2$, where p_i and p_j are frequencies of the i and j allele respectively (Botstein et al. 1980). According to the formula, the higher the number of alleles and more equally frequent they are, the larger will be the PIC. Therefore, binning the null alleles into one category will cause bias in our estimated PIC. We provide a derivation of the maximum bias that can be expected by the assumption that null alleles are all the same:-

$$PIC = 1-\sum_{i=1}^{n}p_i^2-\sum_{i=1}^{n-1}\sum_{j=i+1}^{n}2p_i^2p_j^2. \quad (1)$$

Now let, $i = 1,..\, m,(m+1),..n$, where m and n are the numbers of defined, and the total alleles respectively. If we calculate PIC by putting all the undefined alleles into one category (z), then the restricted PIC is

$$PIC_R = 1-\sum_{i=1}^{m}p_i^2-\left(1-\sum_{i=1}^{m}p_i\right)^2 -\sum_{i=1}^{m-1}\sum_{j=i+1}^{m}2p_i^2p_j^2-2\left(1-\sum_{i=1}^{m}p_i\right)^2\sum_{i=1}^{m}p_i^2. \quad (2)$$

On the contrary, taking all undefined alleles to be different and equally abundant we obtain the full PIC as,

$$PIC_F = 1-\sum_{i=1}^{m}p_i^2-\frac{(1-\sum p_i)^2}{n-m} -\sum_{i=1}^{m-1}\sum_{j=i+1}^{m}2p_i^2p_j^2-\frac{2(1-\sum p_i)^2}{(n-m)^2}\sum_{i=1}^{m}p_i^2, -\frac{n-m-1}{n-m}\left(1-\sum p_i\right)^2 \quad (3)$$

(3)–(2) ⇒

$$PIC_F-PIC_R = 2\left(1-\sum_{i=1}^{m}p_i\right)^2\frac{(\sum p_i)^2}{n}.\left(1-\frac{1}{(n-m)^2}\right). \quad (4)$$

Therefore, the expected maximum difference, for large values of (n-m) from Eq. 4 is then,

$$\Delta PIC = 2(1-\sum_{i=1}^{m}p_i)^2\left[\frac{(\sum p_i)^2}{n}\right].$$

If the undefined alleles constitute only 10% and there are a total of 15 alleles, the difference is ~0.001.

Sequencing of PCR products

The PCR products from two microsatellite markers amplifying in different species were passed through a QIAquick® PCR Purification Kit to eliminate primers, nucleotides, polymerases and salts then run on a 1% agarose/NuSieve® (1:1) gel. Each PCR product was cut from the gel and the DNA extracted and purified using a QIAquick® Gel Extraction Kit. Each sample of the different species was sequenced to compare with the EST sequence from which the marker had been derived.

Results

The cross-species amplification of the markers is summarised in Table 2. All markers were amplified in other species, including those in different sections of the genus, indicating that primer sites were conserved across the genus *Actinidia*. 95% of the markers amplified in taxa of the *Stellatae*, 90% amplified in the *Leiocarpae*, and 83% in the *Maculatae*. In the *Strigosae*, only one taxon was available for testing and in this 18 of the 20 markers amplified, but this may not be representative of the section. Of the markers tested with all 120 genotypes, only one, marker 173, amplified fragments from all genotypes. In most cases where several genotypes from the one taxon were tested, fragments were polymorphic. The least successful marker (marker 237) amplified all genotypes in *A. chinensis* and *A. deliciosa*, and some genotypes in the *Leiocarpae*, but failed to amplify in 19 other taxa, including six from the *Stellatae*. One species in the *Maculatae*, *A. glaucophylla*, with three genotypes, consistently failed to amplify with 9 of the markers; a further 9 amplified in one or two of the three genotypes, but polymorphic fragments were rare, except with marker 173.

The number of fragments generated by the markers was related to the ploidy level of the taxa, the highest number of fragments recorded from one amplification was 12, in a genotype of the octoploid taxon *A. arguta* var. *purpurea* with one of the markers which amplified two loci in *A. chinensis* (marker 226). This marker generated a total of 46 different sized fragments in all the genotypes tested (Table 2) indicating that it was probably amplifying more than one locus in other species as well. Six to eight fragments were regularly recorded from the hexaploid species *A. deliciosa*, and on an average tetraploid species generated more fragments than diploid species. However, even in the diploid taxa, amplification of more than two fragments was not uncommon, and occurred in genotypes of all species. In Table 2 the frequency of polymorphic fragments is indicated but where only one genotype of the taxon was tested, the presence of polymorphism could not be assessed, although in many cases several fragments were generated. Where amplification was successful in only some genotypes of a taxon, it is possible that a homozygous null allele was present, which does not necessarily preclude the marker from being useful in the taxon.

Table 2 Cross-species amplification of markers across taxa

SPECIES	Number of genotypes	118	121	150	154	157	168	171	173	199	200	208	209	211	216	218	220	224	226	237	247
Sect Leiocarpae																					
A. arguta 4*x*	7	4/7	6/7	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	6/7	+ +	0	0	+ +	+ +	+ +	0	+ +
A. arguta purpurea 8*x*	5	3/5	+ +	+ +	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	1/5	2/5	+ +	+ +	+ +	3/5	+ +
A. hypoleuca	1	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	0	+
A. macrosperma	3	+ +	+ +	+ +	2/3	2/3	+ +	+ +	+ +	+ +	1/2*	0	+ +	0	1/3	1/3	+ +	+ +	+ +	0	+ +
A. melanandra 2*x*	1	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+
A. melanandra 4*x*	2	0	0	+ +	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+ +	+ +	+ +	+ +	1/2	+ +
A. polygama	5	+ +	+ +	+	+ +	+	+ +	+	+	+ +	+ +	+ +	+ +	+ +	2/3*	+	1/2*	+	+ +	1/3*	+
A. valvata	2	+ +	+ +	+ +	+	+	+ +	+ +	+ +	+ +	+ +	1/2	+ +	+ +	+ +	+ +	+ +	+	+ +	0	+ +
A. kolomikta	1	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+
Sect Maculatae																					
A. glaucophylla	3	2/3	0	1/2*	0	2/3	2/3	0	+ +	1/3	1/3	+	0	0	1/3	0	2/3	2/3	0	0	0
A. callosa henryi 2*x*	1	+	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	0	+
A. callosa henryi 4*x*	5	+ +	0	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	0	+ +	0	+	+ +	+ +	0	+ +
A. chrysantha	3	+ +	+ +	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	2/3	2/3	+	+ +	+ +	0	+ +
A. indochinensis	3	+ +	0	+ +	+	+ +	+ +	+ +	+	+ +	+	+	+	+	+ +	+	+ +	+ +	+ +	0	+
A. rufa	5	+ +	2/5	2/3*	2/3*	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+ +	1/2*	+ +	+	+ +	0	+
Sect Strigosae																					
A. hemsleyana	1	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+
Sect Stellatae																					
A. chinensis 2*x*	16	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	3/6*	+ +	+ +	+ +	+ +	+ +
A. chinensis 4*x*	3	+ +	+ +	+ +	-	+ +	+ +	+ +	+ +	2/3	+ +	+ +	+ +	-	-	-	+ +	-	-	-	-
A. deliciosa var. *deliciosa*	27	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	25/27	+ +	26/27	+ +	+ +	+ +	4/7*	+ +	+ +	+ +	+ +	+ +
A. deliciosa var. *coloris*	6	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	1/3*	+ +	+ +	+ +	+ +	+ +
A. eriantha	3	1/3	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	1/3	+ +	+ +	+ +	0	+ +
A. fulvicoma	2	0	0	+ +	+	+ +	1/2	+ +	+ +	+ +	+ +	+	+ +	+	+ +	0	+ +	+	+ +	0	+
A. guilinensis	3	+ +	2/3	+ +	+	+	+	+ +	+	+	2/3	+	+ +	+	+	1/3	+ +	+ +	+ +	0	+
A. lanceolata	7	4/6*	1/6*	+ +	+	+ +	+ +	+ +	+ +	5/6*	+ +	5/6*	+ +	+ +	+ +	1/6*	+ +	5/6*	+ +	0	+ +
A. latifolia	3	0	2/3	+ +	+ +	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	1/3	+ +	+	+ +	0	+ +
A. setosa	2	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	1/2	+ +	+ +	1/2	+	+	+ +	+ +	0	+ +

Where not all genotypes of a species showed amplification, the number that did is given against the total number of genotypes tested
A limited number of genotypes was tested in the * samples. The number tested is given. Not tested –
+ indicates amplification; + + indicates polymorphic fragments

Table 3 Summary statistics and the Polymorphism Information Content (PIC) for each of 17 markers which amplified a PCR product scored on a sample of genotypes from a population of different diploid *Actinidia* species

Marker number	Number of individuals			Number of alleles	Allele size range	Microsatellite and unit repeat number	Position relative to the ORF	PIC
	Total	Non-null	2-peak					
118	51	39	25	25	196–230	(CT)9	3′	0.91
121	49	31	21	16	290–319	(CT)17	5′	0.81
150	33	31	17	12	258–298	(CT)17	5′	0.88
157	48	43	24	19	76–129	(AG)17	5′	0.92
168	49	47	21	18	127–173	(CT)17	5′	0.92
171	41	38	22	29	146–203	(CT)19	5′	0.94
173	51	51	23	20	166–201	(AG)20	5′	0.93
199	44	41	21	19	176–207	(CT)16	5′	0.9
200	51	48	20	22	260–308	(AG)15	5′	0.91
208	52	50	16	18	164–194	(CT)15	5′	0.91
209	52	49	27	23	184–208	(AG)16	5′	0.94
211	39	36	16	22	111–149	(CT)16	5′	0.91
218	41	19	7	9	237–259	(CT)16	5′	0.62
220	50	48	21	23	92–160	(CT)16	3′	0.93
224	40	38	21	24	148–189	(AG)16	3′	0.92
237	41	6	4	6	162–180	(AT)16	5′	0.26
247	40	37	13	17	219–293	(AG)16	3′	0.9

The microsatellite type and number of repeat units together with the microsatellite position relative to the open reading frame are listed

The PIC results for each marker are given in Table 3. To estimate PIC, a sample of 54 genotypes was scored for each of 17 markers. Some individual genotype marker data were missing and the actual sample size for each marker is shown. The level of information varied among the markers, as indicated by the numbers of non-null individuals and those that showed two peaks. The range of PIC values was from 0.26 (marker 237) to 0.94 (markers 209 and 171), with 13 of the 17 markers having a PIC value of 0.90 and above. F test and t test results showed that the type of motif (AG vs CT, $P=0.15$) or the number of repeats of the dinucleotide unit ($P=0.92$) did not significantly affect the PIC value

Sequencing of the PCR products from two markers over six species confirmed the presence of the microsatellite repeats in all samples. The number of repeat units was different in all genotypes with marker 173, with a reduction in repeat unit number in 2 genotypes of the same species, and in the other species when compared with the source species (Fig. 1). A similar profile was seen with the primary microsatellite repeat in marker 208. However, a secondary dinucleotide repeat was found to be present in this marker. This second microsatellite also showed polymorphism between the source species and the test species, though in this instance the microsatellite in the source species did not always contain the most unit repeats. A short mononucleotide repeat located between the two microsatellites in the sequence was absent in two of the related species.

Sequences flanking the repeat region were shown to be more variable 5′ of the microsatellite where base changes (markers 173, 208) and deletions and insertions (marker 208) were all observed (Fig. 1). Between the microsatellite and the translational start codon the sequence was much more highly conserved than that preceding the microsatellite in the two examples.

Discussion

The transferability of EST-derived microsatellites from *A. chinensis* and *A. deliciosa* to the other nineteen species from the genus was very high, confirming the conservation of primer sites in transcribed regions of the genome in *Actinidia*. This result was in agreement with that observed by Huang et al. (1998) when they tested cross-amplification of microsatellites originating from an enriched genomic library in nine *Actinidia* species, and in a preliminary report by Weising et al. (1996). The markers generated highly polymorphic fragments in most species where cross-amplification occurred. Polymorphism could be expected considering the great phenotypic diversity observed, comparatively recent cultivation from the wild, and few breeding programmes. The fact that all species so far described are dioecious would also be a factor in maintaining heterozygosity. Where a single genotype was representative of a species no assessment of polymorphism could be made.

Sequencing of the PCR products of two markers across a number of species showed that the microsatellte repeat was present in all the species tested, and of variable length. The chosen repeat region was, in the samples given, longer in the source species (*A. deliciosa* and *A. chinensis*), than in the other species. This finding has been previously reported (Ellegren et al. 1995; Di Gaspero et al. 2000), and has been explained as ascertainment bias in the selection of the loci analysed. Microsatellites chosen on the basis of being highly polymorphic in one species will be those with the longest

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Marker 208
A. deliciosa          Forward primer   TCTCTCTCTCTCTCTCTCTCTCTC----ATCCCCAAAAAAACCCAAAAACC
A. chinensis-mpM                       .........G..............TC--.....-.........A.......
A. chrysantha                          C.C........G.....G......TCTC..............T.....C..
A. eriantha                            .......G.G..........--------............-..A.....--
A. latifolia                           .........G..........--------...............A.....--
A. chinensis-mpD                       ...TGT...G................--.....-.........A....C..

A. deliciosa          AAAAAGCAGTT-CCCGATTTCAACCATTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCAAAGACGTA
A. chinensis-mpM      ...........T...................................------------...........
A. chrysantha         .GC.GG-....-G...CCA...--...................----------------..........G
A. eriantha           ------....-C...............................----------------...........
A. latifolia          ------.....-..........................T........------------...........
A. chinensis-mpD      .....A.....-......AC........................C..------------...........

A. deliciosa          TCCAGAAAAATTGGGATTATTGAGGGACAATGCCGTCGCTAGAAGAG   Reverse primer
A. chinensis-mpM      .............................ATG...............
A. chrysantha         .........-...................ATG.....C...A.....
A. eriantha           .............................ATG..C..T.........
A. latifolia          .............................ATG...............
A. chinensis-mpD      .............................ATG...............

Marker 173
A. chinensis          Forward primer   CCCTCTGGTCTGAAGCCTGAACACCCGAAACGGCTGCCCTTTGCCCCGGAAGTTT
A. chinensis-mpM                       ........G..............................................
A. chrysantha                          ..............-........................................
A. eriantha                            ..TCG..TCA..C.C....TG.T.TGCCCTTT..........C...G........
A. latifolia                           ....G....A..C.A.A...C.C.........C.........C............
A. chinensis-mpD                       ........G..............................................
A. arguta                              .......................................................

A. chinensis          CAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATACTTCTGTAATGGCGGAGAAGCCGCAGCCAGT
A. chinensis-mpM      ..................................---------.G...G...ATG....................
A. chrysantha         ....................-----------------------.T.......ATG....................
A. eriantha           ...............----------------------------.G.......ATG....................
A. latifolia          ...................------------------------.G.......ATG....................
A. chinensis-mpD      ................................-----------.........ATG....................
A. arguta             ................A.-------------------------.........ATG...................A

A. chinensis          GCGAGTTCTATACTGCGGGGTGTGCGGATTACC   Reverse primer
A. chinensis-mpM      ..................C.......A......
A. chrysantha         ..........................A......
A. eriantha           .................................
A. latifolia          ........................G........
A. chinensis-mpD      .................................
A. arguta             .................................
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Fig. 1 Alignment of nucleotide sequences among six *Actinidia* species for two microsatellite loci. *A deliciosa* is the source species in example 1, marker 208, and *A. chinensis* is the source sample in example 2, marker 173. *A. chinensis*-mpM is the female parent and *A chinensis*-mpD is the male parent of the mapping population in which these markers were developed. Identical nucleotides are indicated by *dots*, gaps are indicated by *dashes*. Altered nucleotides are detailed. The sequences were derived directly from PCR products. The *grey* background outlines the putative translational start codon

repeats, and these have generally been found to contain shorter repeats in related species. In marker 208 a secondary, unselected microsatellite can be seen to have an increased number of dinucleotide repeats in two of the related species. While the related species had fewer dinucleotide repeats with marker 208, with marker 173 two of the six related species showed a reduction of an uneven number of bases indicating that deletion or expansion of the dinucleotide repeat was not always regular.

Several authors have reported intraspecific and interspecific allelic size variation at microsatellite loci that was caused by insertions and deletions (indels) in the flanking regions in both animals and plants. Colson and Goldstein (1999) studying *Drosophila* found that only 7 of 19 microsatellite loci showed size variation among species consistent with the occurrence of stepwise mutations in the repeat array, the others showing extensive variability in the flanking region compared to that within the microsatellite itself. Similarly, in a study

of Salmonid, Angers and Bernatchez (1997) discovered indels occurred in non-repeat sequences which independently generated alleles of similar sizes among distantly related groups, or alleles of different sizes within a group. Grimaldi and Crouau-Roy (1997) when characterising the alleles of a (CA)n repeat in humans found a high degree of microsatellite variability was due to variation in the region flanking the repeat. In plants, Matsuoka et al. (2002) found sequencing of microsatellite alleles revealed complex patterns of mutation, including frequent indels in the flanking regions. In their study, 40 of 46 maize microsatellite loci had size distributions that did not fit the model of changes in number of repeat units. Buteler et al. (1999) examined the nucleotide sequence profiles of microsatellite markers in sweet potato and also saw both differences in the repeat number, and indels in the flanking regions. In our study, the size of alleles associated with marker 208 can be seen to be strongly influenced by the presence of indels in the microsatellite flanking region, whereas with marker 173, a single base deletion is present in the flanking region and alteration in the microsatellite repeat number is the major source of polymorphism. Both Matsuoka et al. (2002) and Buteler et al. (1999) worked with microsatellites from genomic libraries while ours were EST-derived, and, being from a coding region, were possibly carrying fewer mutations. Our extensive EST database would suggest that indels in the flanking region are not so common in our markers and the more usual situation is an alteration of dinucleotide repeat number. The majority of our microsatellite loci were 5′ of the open reading frame, and this location may also reduce the incidence of indels in our markers.

As microsatellites are thought to have some regulatory function in the expression of certain genes in the development of neurological disease in humans (Brook et al. 1992; Vincent et al. 2000), it is possible that, while markers may be indicating similar genes, the expression of those genes in different species may give an altered phenotype to that desired. Each marker will need to be tested to determine its usefulness in different genetic backgrounds and species.

Primer pairs frequently amplified more fragments than would be expected from the ploidy level of the genotype tested. While more than one locus has been amplified with some primer pairs, this explanation is not appropriate to all. Genome duplication is common in angiosperms (Wendel 2000), with estimates for the incidence of polyploidy varying from 30 to 80%, and 2–4% of speciation events are also attributed to this phenomenon (Otto and Whitton 2000). Most crop species are thought to be polyploids, and this fact has been thought to confer advantages in that it allowed the development of agronomically important traits. Polyploidisation has been associated with increased fruit size, unusual gene interactions leading to new phenotypes, and the evolution of new crop species (Eckardt 2004). As far back as 1951, Stephens proposed that a relaxation of stabilising selection due to gene duplication would allow the development of novel gene function (Stephens 1951). Analysis of gene expression data showed considerable functional divergence of duplicated gene pairs in a large set of paralogous genes in *Arabidopsis* (Blanc and Wolfe 2004). Kramer et al. (2004) studied the *AGAMOUS (AG)* subfamily of MIKC-type MADS-box genes, which appear to control the development of reproductive organs in both gymnosperms and angiosperms, in fifteen diverse angiosperm species. They found that multiple sub-functionalisation events had occurred after gene duplication which had led to dissociation among the genetic modules and allowed an increase in morphological diversity. Functional divergence of a syntenic invertase gene family in tomato, potato, and *Arabidopsis* has also been reported (Fridman and Zamir 2003). LIN5, a member of a small gene family of apoplastic invertases in tomato, is a quantitative trait locus (QTL) for fruit sugar composition (Fridman et al. 2000). A comparison of the genomic organisation and expression of the gene family in tomato and *Arabidopsis* showed chromosomal segments were syntenically duplicated, but their results indicate that control elements of the orthologous invertase genes had undergone rapid evolutionary change which resulted in divergence in organ expression.

The evidence is increasingly suggesting that functional diploids in *Actinidia* are, in fact, at least tetraploid. McNeilage and Considine (1989) suggested that the genus could have become a stabilised tetraploid from ancestors with $x = 14/15$. While this feature would not affect the breeding capability of species at recognised ploidies, it may have an influence on the accuracy of marker/trait associations across ploidy levels.

In the final analysis, successful marker-assisted selection will depend on a number of qualifying parameters. Markers will need to be as close to the gene as possible for utility across all populations, and they will have to be shown to be identifying the genetic component of the desired trait. The indication from the sequencing results that the amplification products are marking a related gene in species of *Actinidia* other than the marker source species is encouraging in assessing the potential of the markers for breeding. However, this homology will have to be tested with each individual marker before it is used in a breeding population.

Acknowledgements We thank Hanna-Mari Tervo and Zhening Zhang for excellent technical assistance, and Bhawana Nain for sequencing data. This research was partially funded by the New Zealand Foundation for Research, Science and Technology (CO6X0213) and by the HortResearch Royalty Fund.

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