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G. P. Gill · C. F. Harvey · R. C. Gardner
L. G. Fraser

Development of sex-linked PCR markers for gender identification in *Actinidia*

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Abstract Two sex-linked random amplified polymorphic DNA (RAPD) markers identified from *Actinidia chinensis* were converted into sequence-characterised amplified regions (SCARs) for the large-scale screening of *Actinidia* breeding populations. Initial SCAR primers converted one RAPD (SmX) into a dominant marker, but the other (SmY), which was potentially more useful because of its linkage to the male determining 'Y' locus, failed to retain polymorphism. This difficulty was overcome by cloning and sequencing the alternate 'allele' from female plants, and then designing 'allele'-specific primers that utilised nucleotide differences between the sexes. Using a quick squash-blot method of DNA extraction, the SCAR primers were tested in 120 *A. chinensis* plants to determine their gender. The system is now in use for large-scale screening of seedling populations in the *Actinidia* breeding programme. The sex-linked SCAR primers also functioned with plants from some other geographically separate accessions of *A. chinensis* and with plants in the closely related polyploid species *A. deliciosa*, but did not amplify a sex-linked band in more distantly related species of *Actinidia*.

Key words *Actinidia* · RAPD · SCAR · Sex-linked markers · Marker-assisted selection

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G. P. Gill · C. F. Harvey · L. G. Fraser (✉)
The Horticulture and Food Research Institute of New Zealand Ltd.,
Mt Albert Research Centre, Private Bag 92169,
Auckland, New Zealand

R. C. Gardner
Plant Molecular Biology, School of Biological Sciences,
University of Auckland, Auckland, New Zealand

Introduction

The genus *Actinidia* contains about 60 species of woody vines which have originated in China and neighbouring countries. *Actinidia deliciosa* (A. Chev) C.F. Liang et A.R. Ferguson var. *deliciosa* cv. Hayward (kiwifruit) is a major fruit crop that is cultivated extensively in New Zealand, Italy, Chile, Greece, and other temperate to sub-tropical areas. Because the kiwifruit industry is based on a single cultivar, the need for new cultivars has been recognised and breeding programmes have been established. Species of *Actinidia* other than *A. deliciosa* have economic potential, and diversity in characters such as fruit colour, flavour, skin texture, fruit size and vine growth habit offer the opportunity for improvement and variety through selection and interspecific hybridisation (Harvey et al. 1995; Ferguson et al. 1996). *Actinidia chinensis* Planch., a species containing both diploid and tetraploid races, was available for testing in New Zealand. It is closely related to the hexaploid cultivated species *A. deliciosa* to which it is thought to have contributed at least one genome (Crowhurst et al. 1990; Crowhurst and Gardner 1991; Atkinson et al. 1997; Yan et al. 1997).

All species in the genus *Actinidia* are functionally dioecious and have no distinguishing cytological or vegetative features to identify the sex of seedlings. Plants reach reproductive maturity in 3–5 years, with male plants making up approximately 50% of breeding populations (Testolin et al. 1995). In populations where fruiting selections are desired, the requirement to grow seedlings to maturity before eliminating the unwanted males represents a considerable cost in plant maintenance. A method for identifying the sex of seedlings would therefore increase the efficiency of a breeding programme by permitting the early elimination of unwanted plants.

The development of the polymerase chain reaction (PCR) fingerprinting techniques, random amplified

polymorphic DNAs (RAPDs) (Welsh and McClelland 1990; Williams et al. 1990), amplified fragment length polymorphisms (AFLPs) (Lin and Kuo 1995), and simple sequence repeats (SSRs) (Powell et al. 1996) has accelerated the detection of markers in plant genomes. By combining RAPDs with bulk segregant analysis (BSA), where two pooled DNA samples are formed from plants which have similar genetic backgrounds but differ in one particular trait, markers linked to that trait can be identified (Michelmore et al. 1991). This method has been employed to find four RAPD sex-linked markers using 60 random primers in *Silene latifolia* (Mulcahy et al. 1992), and a single sex-linked marker in *Pistacia vera* after screening 700 primers (Hormaza et al. 1994). We have used a similar strategy and screened 500 random primers in female and male bulk-DNA preparations derived from siblings to identify two sex-linked RAPD markers in *A. chinensis*. One marker, SmX, was inherited by female progeny from the male parent, and the other, SmY, was inherited by male progeny from the male parent. Inheritance studies of the sex-linked RAPD markers have demonstrated that an XY-type system of sex determination functions in *Actinidia* with the male being the heterogametic sex (Harvey et al. 1997).

Sensitivity to reaction conditions and the requirement for high-quality DNA can hinder the use of RAPD markers for routine analysis. To overcome these difficulties RAPDs can be converted to sequence-characterised amplified regions (SCARs) (Paran and Michelmore 1993). SCAR primers amplify single bands corresponding to genetically defined loci, are less sensitive to reaction conditions, and have the capability of becoming co-dominant markers. They are similar to the sequence-tagged sites (STSs) used by Olson et al. (1989) as landmarks for physical mapping of the human genome, and have been used extensively in crop species for mapping and marker-aided selection (Timmerman et al. 1994; Schachermayr et al. 1994; Williamson et al. 1994; Nair et al. 1995; Witsenboer et al. 1995; Ohmori et al. 1996; Procunier et al. 1997). SCARs can retain the original polymorphism defined by the RAPD marker by appearing as a dominant marker or as a co-dominant length polymorphism. Often SCAR primers will amplify a same-sized alternate 'allele' resulting in a loss of the original RAPD polymorphism. Digestion of the products with restriction enzymes, increased annealing temperatures, and increased resolution to identify small size differences, are methods that can be used for retrieving a polymorphism (Paran and Michelmore 1993).

This paper describes the conversion of two sex-linked RAPD markers identified from *A. chinensis* into SCARS. One of these markers (SmY) required the strategy of cloning and sequencing the alternate allele from the female parent for designing primers that utilised nucleotide differences between the sexes. This marker was then applied to a population of *A. chinensis*.

Materials and methods

Plant material

The parents and F_1 siblings of a diploid *A. chinensis* family used to identify the RAPD markers SmX and SmY (Harvey et al. 1997) provided material for marker development and analysis. Plants of other *A. chinensis* accessions from different geographical areas, as well as *A. deliciosa*, *A. eriantha* Benth., *A. arguta* (Sieb. et Zucc.) Planch. ex Miq., *A. latifolia* (Gardn. et Champ.) Merr. and *A. rufa* (Sieb. et Zucc.) Planch. ex Miq., were obtained from the Hort-Research orchards at Te Puke or Kumeu, New Zealand.

Genomic DNA extractions

Actinidia genomic DNA for the gender identification test was extracted by a modification of the method described by Langridge et al. (1991). Circles of a Hybond™-N^+ positively charged nylon membrane (Amersham) were cut with a paper punch and inserted in the wells of a 96-well ELISA plate. Hybond circles adhered to the bottom of the wells when a drop of acetone was used to soften the plastic. The trays were allowed to dry and excess acetone to evaporate. Pieces of tissue about 3–5 mm in diameter were taken from young leaves about 1 cm in length and placed in the wells on the circles of Hybond. An aliquot (20 µl) of 1 M NaOH was added and the plant material was crushed on the membrane using a glass rod. Using a multi-channel pipette, 50 µl of high-salt buffer (1.5 M NaCl, 0.5 M Tris.Cl pH 7.0, 1 mM EDTA) was added, and removed after 1 min. A 100-µl aliquot of low-salt buffer (1 mM EDTA, 10 mM Tris.Cl pH 7.5) was then added and also removed after 1 min. A final wash with sterile distilled water (100 µl) was added and then removed. The DNA adhering to each membrane was eluted in 70 µl of water when the tray was heated for 3 min in an oven at 95°C. The extracts were then transferred from the processing wells into clean wells and 5 µl used directly for PCR, or, alternatively, the preparations could be frozen at −20°C until required. Frozen preparations were used within 10 days in PCR reactions.

Larger, purer genomic DNA preparations were made using a CTAB method (Harvey et al. 1997) or a DNeasy™ (Qiagen) kit.

Polymerase chain reactions (PCRs)

PCR conditions for the SCAR primers were identical to those used for the RAPD reactions (Harvey et al. 1997), except that the concentration of SCAR primers was 0.2 µm and the annealing temperature was optimised for each set of primers (see Table 1). A touchdown programme where the annealing temperature was dropped by one degree every two cycles, from 58°C to 55°C for the first eight cycles, followed by 30 cycles at 54°C, was used for the large-scale screening with SmY. When testing with DNA prepared by the rapid squash-blot method, 1% non-fat milk powder termed Blotto (bovine lacto transfer technique optimiser) was included in the PCR to minimise inhibition by contaminating substances (De Boer et al. 1995).

Eight available primer sets designed in *Actinidia* were multiplexed with SmY for evaluation as potential positive controls.

Cloning and sequencing of RAPD markers

RAPD markers SmX and SmY were amplified using DNA from the male parent of the original *A. chinensis* family. Polymorphic bands were resolved on a 1.2% low-melting-point agarose gel before being excised and purified using the Wizard™ PCR Preps DNA Purification System (Promega). The purified product was cloned into

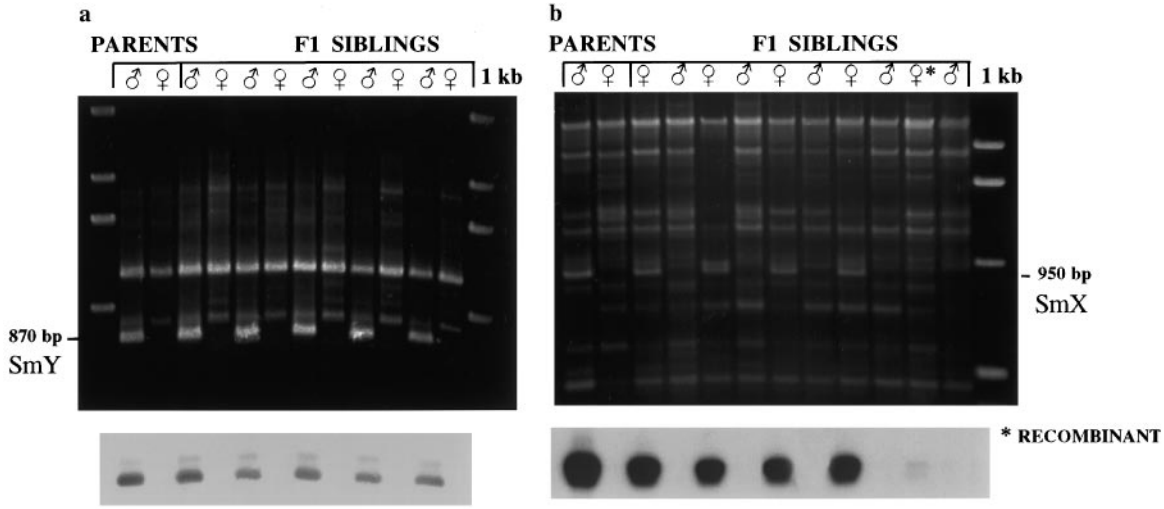

Fig. 1a,b RAPD gels and DNA gel-blot hybridisations. **a** Primer OPAI-12 showing a sex-linked RAPD band in the male parent and male progeny. DNA gel-blot hybridisation of cloned band shown below. **b** Primer OPAL-20 showing a sex-linked RAPD band in the male parent and female progeny. DNA gel-blot hybridisation of cloned band shown below

a pGEM-T vector (Promega). The identity of the cloned product was verified by hybridisation to a DNA gel-blot of RAPD products segregating for the band of interest. Sequencing was performed by the dideoxynucleotide chain-termination method using an Applied Biosystems robotic workstation and a 373 A sequencer. The RAPD primers used in the amplification of SmX and SmY were OPAL-20 and OPAI-12, synthesised by Operon Technologies (Alameda, California).

Results

Cloning and sequencing of SmX and SmY

The SmX and SmY RAPD marker bands were cloned into the pGEM-T vector to identify sequences to be used in the design of SCAR primers. RAPD gel-blots probed with the cloned markers hybridised strongly to the segregating band of interest (Fig. 1). Additional hybridisation occurred to other RAPD products that were sex-linked but were not visible by ethidium-bromide staining. One female recombinant plant for SmX (band absent) showed a very faint hybridisation signal to two RAPD products of similiar size, although no corresponding RAPD band could be visualised. The sequence of each sex marker was obtained for at least 250 bp of single-stranded DNA in both forward and reverse directions.

SCAR analysis

Four SCAR primers, SmXf and SmXr, SmYf and SmYr, were produced that consisted of the original RAPD primer followed by the next 12–13 nucleotides of the sequenced bands. The sequences, annealing temperature, and product size of SCAR primers are listed in Table 1.

The SCAR primers SmXf and SmXr, amplified an intense band at a 60°C annealing temperature in female siblings of the original family, and a faint band in male

Table 1 Sequences, product sizes and annealing temperatures of SCAR primers for SmX and SmY

Primer	Sequence[a]	Annealing temperature	Band size (bp)	Polymorphism
SMXf	AGGAGTCGGAGAGAGTAGAGAAG	68°C	950	Dominant[b]
SMXr	AGGAGTCGGATGACCGTTGGTGA			
SMYf	GACGCGAACCACCCACATTTGAG	65°C	870	None
SMYr	GACGCGAACCCGGCAAGTCGAAC			
SMYf1	TCGCAATTCGTTAGGGATGATGCG	58–54°C touchdown	770	Dominant
SMYr1	CATAATCAACCATCCATAAAAACCAT			

[a] The underlined sequence represents the progenitor RAPD primer. Operon primers were OPAL-20 (SMX) and OPAI-12 (SMY)
[b] The lower annealing temperatures utilised (55–60°C) gave no detectable polymorphism

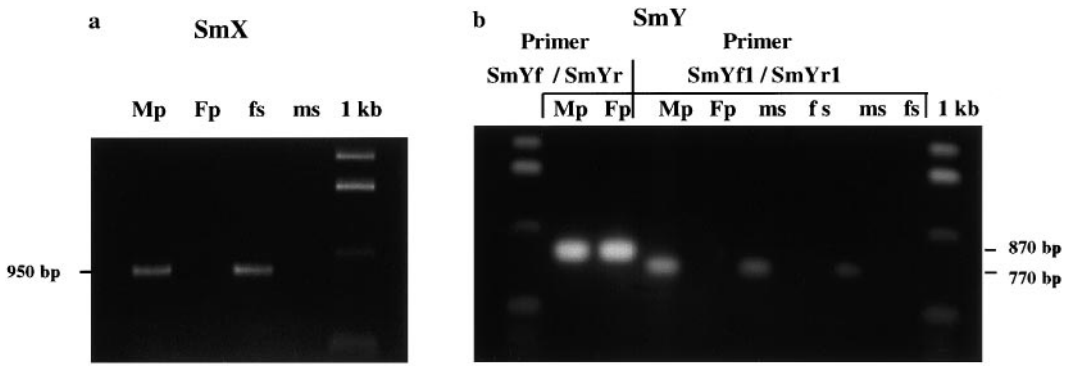

Fig. 2a Amplification of genomic DNAs from *A. chinensis* using SCAR primers SmXf and SmXr at an annealing temperature of 67°C. *Mp* male parent, *Fp* female parent, *fs* female sibling, *ms* male sibling. **b** Amplification of genomic DNAs from *A. chinensis* using initial SCAR primers SmYf and SmYr and allele-specific primers SmYf1 and SmYr1

siblings. When the annealing temperature was raised to 67°C these primers amplified a dominant band in females only (Fig. 2 a).

At an annealing temperature of 65°C initial SCAR primers SmYf and SmYr amplified a band of the same size in the DNA of both male and female plants (Fig. 2 b). Increasing the stringency by the use of a Mg concentration gradient allowed the faint amplification of a band in male plants only, but this result was highly unreliable. Digestion of the male and female amplification products by 15 common restriction enzymes with 4-bp recognition sequences failed to retrieve a polymorphism between the male and female loci.

Generation of sex-distinguishing SCAR primers for SmY

The primers SmYf and SmYr were used to amplify products from male and female *A. chinensis* parents and male and female *A. deliciosa* var. *deliciosa*. Two clones from each plant were partially sequenced to give 250 bp of consensus sequence in both the forward and reverse directions. The sequence from one male *A. chinensis* clone showed nine nucleotide differences in the sequenced region when compared to the sequence from the female (Fig. 3). SCAR primers SmYf1 and SmYr1 were designed to amplify a male-linked dominant band by utilising these nucleotide differences between the sexes of *A. chinensis* (Figs 2 b, 3). These specific primers for SmY (Table 1) were designed to have critical nucleotide mismatches placed at the 3′ end of the target amplification band (Fig. 3).

There were no sequence differences between the clones obtained from female *A. deliciosa* var. *deliciosa* and female *A. chinensis*. Some clones obtained from male *A. deliciosa* also gave an identical sequence to the female-derived clones. However, the primers designed to give the male-specific band in *A. chinensis* amplified a male-specific band in *A. deliciosa*, although the *A. deliciosa* product was not amplified to the same extent. Ten clones containing PCR products amplified with initial primers SmYf and SmYr from the male *A. deliciosa* were then tested using the modified primers SmYf1 and SmYr1. Two clones amplified a strong band with the modified primers. The sequences of these clones showed that they were similar to the *A. chinensis* 'Y alleles' except for 2 bp situated in the new primer region which were different (Fig. 3).

SmX and SmY in accessions and species of *Actinidia*

Two individuals of each sex were tested from each accession or species. Male plants in five out of nine accessions of *A. chinensis* showed a sex-linked band with the specific primers SmYf1 and SmYr1. In the other four *A. chinensis* accessions the marker was either absent or gave a faint, unreliable, band. Female plants, except for a few known recombinant plants which had been previously identified (Harvey et al. 1997), gave no band.

In *A. deliciosa* var. *deliciosa* SmX was amplified with the SCAR primers SmXf and SmXr but was no longer polymorphic between the sexes, even at the higher annealing temperature of 67°C. The male-linked primers SmYf1 and SmYr1 amplified a dominant band in a small number of males from three different accessions of *A. deliciosa* but not in any of the female genotypes tested.

DNA of individuals from *A. eriantha*, *A. arguta*, *A. latifolia*, *A. setosa* and *A. rufa* did not amplify a sex-linked band with any of the SCAR primers for either SmY or SmX.

Testing SmY in an *A. chinensis* population

The sex-linked SmY SCAR primers were tested in a population of 120 *A. chinensis* plants whose gender

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Forward
                                                          5'
SMYy   1  GACGCGAACCACCCACATTTGAGATCGTGCGATCCAAATCGCAATTCGTT
SMYx      ...................................................
                    3'
SMYy 51   AGGGATGATGCGAATTGTGGTAACCCTGGTTAGAACTTATTCATAGCAAA
SMYx      .......G...A.......................................

SMYy 101  GTTTTGAAAATTGTTTGTTCTCAAAAAGAATAATTAAGTAAAAGTTTATC
SMYx      ................................C..................

SMYy 151  TTATTTCATCTTACCTCGCCTCTATGGTATCAGTGGCGGACGCAGGAATT
SMYx      ...................................................

SMYy 201  ATGGTTAGAGGGGGCCAAATTATACATTTTCTATAGA
SMYx      ...................................


Reverse

SMYy   1  GACGCGAACCCGGCAAGTCGAACCCCACCAAGATGGCAGAGCTCAAGCTA
SMYx      ...................................................
                        5'                          3'
SMYy  51  TAGAAAACCTGCTCATAATCAACCATCCATAAAAACCATACACCATTAAA
SMYx      ..............C........G.........T...A..............

SMYy 101  CCAGAATCAAACACAATCTATTCATTCACCAATTCTAAATCTCACCAAAA
SMYx      T..................................................

SMYy 151  AGCAATCATGCAATGGGGAATATCATAGTCAATGGGTTTGTATTCAACCA
SMYx      ............................C......................

SMYy 201  GCAATAATATAAAAGATACTCCATCAGATACATCCATCTA
SMYx      ......................................
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Fig. 3 Partial sequences of SMY cloned fragments from *A. chinensis* and *A. deliciosa* displaying nucleotide differences in the 'Y' and 'X' 'alleles'. The regions *underlined* indicate the position of the successful male-linked primers SmYf1 and SmYr1. The nucleotides in *bold* were different between *A. chinensis* and *A. deliciosa* male-derived clones. The sequence from the *A. deliciosa* female-derived clones was identical to the 'X allele' from *A. chinensis*

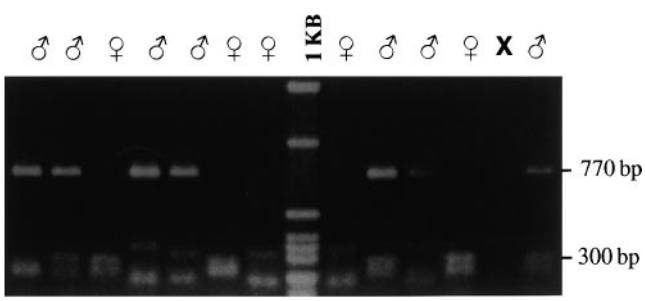

Fig. 4 SCAR SmY amplifying a band in males only, with primers SmYf1 and SmYr1, used with squash-blot DNA in a test population of *A. chinensis*. The variable band(s) at about 300 bp is a positive control amplified by microsatellite primer 721. *Lanes marked with X* are DNA extractions or PCR failures

was confirmed by flower morphology at their first flowering 1 month later. The first application of the test resulted in the gender of 92 plants (77%) being accurately confirmed, with 50 plants being identified as males and 42 as females. Of the first 120 tests, 28 were ambiguous, but 13 of these were resolved on applying the test a second time. This gave a success rate of 88%. Further testing showed that the remaining 12% was composed of plants which were either recombinant for the marker (3%) or were the result of DNA extraction failures (9%).

In order to confirm that the PCR reaction had been successful, a positive control functioning at the same annealing temperature as SmY was sought. The most promising was microsatellite primer 721 (Weising et al. 1996). This positive control would identify female plants in cases where DNA extraction or PCR had failed (Fig. 4). The amplified band was usually about 300 bp, but was sometimes small enough to be confused with a primer-dimer band.

Discussion

SCAR primers will occasionally amplify an alternate, same-sized, 'allele' where polymorphism can not be recovered by the use of restriction enzymes, increased annealing temperatures, or increased resolution for small size differences. Paran and Michelmore (1993) reported one out of nine, and Witsenboer et al. (1995) one out of eight, SCARs where a polymorphism could not be recovered. In the former case there was a single base-pair difference between alleles and a suitable restriction enzyme was not commercially available. When considerable experimental effort has identified a useful marker, the failure to retrieve a polymorphism is a significant loss. An amplification refractory mutation system (ARMS) (Newton et al. 1989) was designed to specifically amplify human-disease alleles differing by as little as 1 bp. Primers that have particular mismatches placed at the 3′-terminus of the targeted amplification site have been shown to yield an allele-specific product even where alleles differed by as little as 1 bp (Kwok et al. 1990; Huang et al. 1992). Witsenboer et al. (1995) was able to construct a dominant SCAR marker by shifting the primer position 5–8 bases internal to the original RAPD primer, thus relocating unknown nucleotide differences near the 3′ end.

In the development of the SCAR SmY we have taken the approach of cloning and sequencing the products of both 'alleles' to allow critical nucleotide differences to be deliberately situated at the terminal 3′ end of the primer. This option may be quicker and less expensive than the use of restriction enzymes to retrieve polymorphism, especially if mass screening of plants is the ultimate objective.

The success of the gender identification test demonstrates that the marker has considerable practical application, although it cannot be used in all *A. chinensis* accessions. Cost is critical in the application of such a test and an unambiguous answer after one test contributes to keeping costs down, both the direct costs of the test and the indirect costs of maintaining unwanted plants in the orchard. To reduce the possibility of confusing female plants where the marker is absent, with failures of PCR or DNA extraction, a positive control band which did not interfere with the marker

band was sought. Theoretically, multiplexing SmY with SmX would seem desirable but this was not possible because of the difference in their optimum annealing temperatures (Table 1). The positive-control microsatellite primer 721 (Weising et al. 1996) functioned satisfactorily but will be replaced with additional X-linked sex markers as they are found. In the cases where the first test was ambiguous the most frequent problem was the DNA extraction. Freshness of the plant material, and speed during the extraction to remove degrading or oxidising material from the DNA, were critical factors and a constraint on the handling of large numbers of samples. *Actinidia* material from older plants contained phenolics and polysaccharides which could contaminate DNA from the rapid technique and make it unsuitable for PCR. The addition of non-fat milk powder (BLOTTO) to the PCR mixture was found to be beneficial in reducing the effects of PCR inhibitors which were present in the squash-blot DNA extractions. Seedling material was found to oxidise less than older material and was therefore preferable for testing. As the advantage of gender identification is greatest when applied at the earliest possible stage, the DNA extraction protocol has now been optimised for seedling material using either cotyledons or first true leaves, and handling of larger numbers is possible. The gender identification test is currently being applied to 3000 seedlings in the Institute's breeding programme.

Intrinsic sources of error are also present during the use of the male marker SmY. During the elimination of male plants any female recombinants would be discarded. We have previously shown that this should be about 2.5% of plants (Harvey et al. 1997). The rate of recombination between the marker and the sex determination locus in this test was 3%, which is comparable with our earlier result. Where a male is a recombinant it would be kept and grown in the field thus reducing the cost-efficiency of the test.

RAPD DNA gel-blots probed with the marker of interest faintly hybridised to other products that were sex-specific, but did not correspond to visible segregating bands. These could be alternate alleles or sequence-related bands. Genomic DNA gel-blot analysis of both markers (data not presented) revealed that both SmX and SmY were moderately to highly repetitive sequences, as is commonly found with markers derived from RAPDs (Paran and Michelmore 1993), therefore excluding their use as hybridisation probes. The partial sequences obtained for SmY and SmX showed no homology to any known sequences and did not contain any recognisable open reading frames, so are probably in non-coding regions. If we assume that the region amplified by SmY is on the 'Y' chromosome of an XY pair, it is apparent that a very similar sequence is present on the 'X' homologue. The similarity of sequence in this region extends to *A. deliciosa*. The 2-bp differences between the SmY 'Y alleles' of *A. deliciosa* and *A. chinensis* were located in the SmYf1 and SmYr1 primer sites. This could explain why the SmY product was less intensely amplified, but was still able to identify males in this species. However, the small number of clones sequenced, and the possibility of PCR or sequencing errors, must be considered. In a species there may be a number of small sequence differences in non-coding regions. Sequence information from a greater number of plants would be needed to resolve this question.

Previous work has suggested that the diploid *A. chinensis* has contributed to the hexaploid *A. deliciosa* genome. (Crowhurst et al. 1990; Crowhurst and Gardner 1991; Atkinson et al. 1997; Yan et al. 1997). A further suggestion from our work is that *A. chinensis* is a diverse species, and genomes from only some geographic areas have been involved in the evolution of *A. deliciosa*. The widespread distribution and variability of *A. chinensis* in China and the more restricted distribution of *A. deliciosa* also support this suggestion.

When cloning the SmY SCAR product in the hexaploid *A. deliciosa* male using the SmYf and SmYr primers, a larger proportion of 'X allele' clones compared to 'Y allele' clones was obtained. Although we cannot be certain that the PCR and cloning technique are sufficiently quantitative to reflect the allele proportions, this result supports the proposal that the chromosomal constitution of the hexaploid male is XXXXXY (or possibly XXXXYY), the Y chromosome bearing a locus which is male-determining despite the presence of several X chromosomes.

SmY has proved to be a useful tool for the gender determination of seedlings in certain *A. chinensis* breeding populations. Further sex-linked markers, which could be used in all accessions and species of *Actinidia*, are being sought using the high-throughput capability of fluorescently labelled AFLPs analysed on a ABI 377 DNA sequencer.

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