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Research Report

A molecular protocol for Early Sex Discrimination (ESD) in *Actinidia* spp

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Abstract. Dioecism and an extended juvenile phase of 3–7 years in kiwifruit hinder the progress in breeding new cultivars. The identification of fruit-bearing females at an early stage of growth is crucial for breeders. Consequently, molecular markers have become a key tool for identifying female and male plants at an early stage of development. Several efforts were made to identify PCR-based sex linked markers in *Actinidia*; however, those markers are characterized by a highly polymorphic nature affecting the result of the screening reliability, suggesting the need of more suitable, stable markers, characterized by a consistent transferability among genotypes and species. The main goal of this work was to develop a method for the ultimate discrimination of females from male plants at an early stage of growth using sex-linked markers. We developed an Early Sex Discrimination molecular Test (ESD Test) that allows the discrimination of male and female plants using a simple PCR amplification test. We demonstrate that the test could unequivocally identify the gender of an unknown sample both in the most commercially important species *A. chinensis* and in further 13 *Actinidia* species tested with the exception of *Actinidia latifolia*, where markers fail in gender discrimination. Male genotypes could be easily identified and discarded reducing the cost of a breeding program.

Keywords: Molecular markers, kiwifruit, marker assisted selection (MAS), plant breeding

1. Introduction

The genus *Actinidia* Lindl. includes about 54 species of climbing plants, all dioecious, originating mainly in central and southern China [1]. Kiwifruit is one of the most recently domesticated fruit crops. The development of new cultivars showing interesting traits for the consumer, e.g., taste, appearance, and health components, has driven the commercial success of this fruit and kiwifruit has become an important horticultural crop over the years, firstly in New Zealand and subsequently in many other countries such as Chile, China and Italy [1, 2].

The kiwifruit is dioecious, with male and female flowers carried on different plants. Male individuals, bearing staminate flowers with numerous stamens and a rudimentary ovary lacking ovules, stand out from female individuals that carry well-developed ovaries but do not produce viable pollen. Gender is monofactorial and it is apparently controlled by a single Mendelian determinant, with the female sex being homogametic (XX) and the

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male sex heterogametic (XY) [3–5]. Any cross between female and male genotypes usually produces female and male offspring in the ratio of 1 : 1 whatever the ploidy level [3]. Male plants are a waste of land and resources because breeders are usually focused on selecting fruit-bearing individuals. The situation is exacerbated by the long generation cycle which is spread at least between three seasons, interrupted by winter dormancy [6]. Development and introduction of improved cultivars may require many breeding cycles and decades [7]. The management cost of relatively large cross populations over several years is a limiting factor in any woody species-breeding program, particularly in fruit crops like kiwifruit, where expensive support structures are required. Since kiwifruit breeders are seldom interested in selecting males in breeding programs, a great effort has gone into the search for sex markers. The ability to discard male seedlings at a very early stage through the analysis of associated markers would save labor and space in the orchard.

The sex determinant was mapped in the sub-telomeric region of chromosome 17 [8], which is the pseudo-molecule 25 in the more recent genome assembly of *Actinidia chinensis* var. *chinensis* (genotype Red5) [9]. The suppression of recombination in this region is the key feature of sex chromosomes, allowing the two chromosomes of the pair to evolve separately and the multiple genes involved to be inherited as a single genetic determinant, so that only male and female progeny are produced [10–12]. Therefore, the finding of markers very close to the sex determinants is challenging and it is also difficult to establish the order and the distances that separate markers and sex-genes, considering also the relatively small size of the populations studied. The first attempt to find markers linked to gender was based on the combination of RAPD markers and bulk segregant analysis (BSA). The screening of 500 random primers allowed the discovery of two sex markers, which were then transformed into three SCAR (Sequence-Characterized Amplified Regions) markers, by cloning and sequencing the original RAPD and designing allele-specific primers [13]. These markers were soon deployed routinely in marker-assisted kiwifruit breeding, to eliminate male plants at the seedling stage [14]. The closest gender markers reported up to now are the markers SmX, UDK096, SmY1 and Ke225 spanning 4 cM in the region where the sex phenotype maps. The last one is now preferred in breeding programs in New Zealand (http://www.plantandfood.co.nz), but the primers have not yet been published. On the other hand, markers such as SmX and SmY1 were used in the past but they are not always transferable from one cross population to another and from one species to another and if they are, they sometimes lose their polymorphism [4, 13]. More recently, SNP-based genetic maps have been published that allowed the anchoring of sex-surrounding scaffolds onto the genome assembly and the isolation of new SSR markers from those scaffolds. These new SSR markers could discriminate between male and female progeny in most crosses analyzed [15, 16] whatever the ploidy level of the cross parents. Other markers named A001, A002 and A003 were also successfully amplified in *A. rufa* and interspecific crosses with *A. chinensis* var. *chinensis* [16].

However, because of the highly polymorphic nature of SSR markers, the sex-linked alleles were not always of the same length in the different crosses assayed. Therefore, the identification of the alleles carried by the cross parents is necessary for the analysis of the allele associated with the gender in the progeny. These markers are therefore not suitable for screening germplasm collections where the parentage of accessions is not known in advance [15, 17]. Consequently, new markers associated with the sex determinants that are more appropriate, stable, and characterized by greater transportability among genotypes and species are necessary.

Recently, the two Y-encoded sex determinants in kiwifruit were described by Akagi and co-workers. The Shy Girl (*SyGI*) gene, which is a Y-encoded cytokinin response regulator that acts as the suppressor of female development [18] is expressed in developing flowers, specifically at the surface of the rudimentary carpels of male flowers. The Friendly Boy (*FrBy*), a fasciclin-like protein which would maintain male fertility, exhibits strong expression in tapetal cells [19]. Moreover, *FrBy* acts for the maintenance of male (M) functions, independently of *SyGI*. These two sex determinants are located at an estimated distance of 500 kb in the specific region of the Y chromosome while they are absent in the corresponding chromosomal female region.

In the present study, we set up an early sex discrimination molecular test (ESD test) using primers designed on the *SyGI* and *FrBy* genes that can discriminate between male and female plants with a simple signal of presence/absence both among the species and on genotypes within different ploidies. We also include the

housekeeping Ankyrin repeat domain-containing protein (*Ank*) marker that acts as internal control to test successful PCR amplification.

We developed a simple experimental protocol, which requires only a crude leaf extract, thus reducing the time needed for DNA extraction. The amplicons can be separated on both agarose gel and capillary sequencer. The experimental protocol can be used for rapid selection of female genotypes, reducing the management costs of breeding programs.

2. Materials and methods

2.1. Plant materials

Young leaves from 43 different male and female *Actinidia* genotypes were collected from the kiwifruit repository of the experimental farm "A. Servadei" of the University of Udine, Italy. We selected 13 unrelated genotypes from *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa* and 30 genotypes from 14 other *Actinidia* species. The genotypes collected are listed in Table 1. Young leaves were collected as duplicate samples during the growing season. In addition, leaves from 31 individuals were collected from two different cross-populations named Ac565 (cross *A. chinensis* var. *chinensis* 'Jintao' X Ac442 male pollen mix) and Ac567 (cross Ac0171.76 'Soreli' (*A. chinensis* var. *chinensis*) × A0074 male 'Cornell' (*A. arguta*)).

2. 2 DNA extraction methods

Genomic DNA of the first replicate of samples was extracted using NucleoSpin® Plant II kit (Macherey-Nagel) starting from lyophilized material ground using the Tissue Lyser (30 Hz × 2 min × 2v). Briefly, plant samples after homogenization were extracted with Lysis Buffer PL1, which is based on the CTAB (cetyltrimethylammonium bromide) extraction method. DNA quantification was performed using the Nanodrop ND-100 spectrophotometer.

The second replicate of samples was used to test a quick protocol of extraction (Tyrone Possamai 2021, pers comm) which produces a crude extract suitable for DNA analysis in less than one hour. Briefly, lyophilized leaf samples were ground using the Tissue Lyser (30 Hz × 2 min × 2v). Then, 450 µl of lysis buffer were added and the samples were thoroughly mixed. The samples were then incubated for 15–20 min at 65°C. Next 130 µl of precipitation buffer were added to the samples, they were mixed for 15 sec, put on ice for 10 min and then centrifuged for 20 min at 15 000 × g. Finally, the clear supernatant was collected for analysis.

Lysis Buffer is the AP1 lysis buffer (Qiagen) based on SDS extraction method. This buffer can be made in the laboratory by mixing 0.5% W/V SDS (sodium dodecyl sulfate), 8% W/V PVP-10 (optional), 250 mM sodium chloride, 25 mM NaEDTA and 200 mM Tris – HCl, pH 7.5. Precipitation buffer is the P3 precipitation buffer (Qiagen) containing 5M potassium acetate.

2.3. Targets amplification using different methods

Primers were designed within the coding region of the Ankirin repeat domain-containing protein (*Ank*) in the LG1 of Red5 reference genome (18,539,780–18,539,855, 18,540,915–18,541,078 bp) and Friendly Boy (*FrBy*) genes using Primer 3 [20]. Primers specific for Shy Girl (*SyGI*) gene were published by Akagi and co-workers [18]. Primer sequences are reported in Supplementary Table S1. The primer pairs were selected aiming for the amplification of three size-distinguishable PCR products. PCR reactions for the amplification of the three targets were performed using the 5 PRIME HotMaster® Taq DNA Polymerase (QuantaBio) on the samples extracted with NucleoSpin® Plant II kit (Macherey-Nagel). The PCR reaction recipe is given in Tables 2a and 2b. The PCR thermal conditions were 94°C for 2 min, followed by 30 cycles at 94°C for 20 sec, 50°C for 20 sec and 65°C for 30 sec, and a final extension at 65°C for 5 min. The amplification success was verified running PCR products on a 1.5% agarose gel.

Table 1
List of genotypes used in this work

Genotype	Gender	Species
A0181	M	*A. chinensis* var. *chinensis*
A0202.32	F	*A. chinensis* var. *chinensis*
A0182	M	*A. chinensis* var. *chinensis*
Ac453.004	F	*A. chinensis* var. *chinensis*
Ac178.15	M	*A. chinensis* var. *chinensis*
Ac175.98	I	*A. chinensis* var. *chinensis*
A0185.1 Ecor	F	*A. chinensis* var. *deliciosa*
A0185.2 Ecor	M	*A. chinensis* var. *deliciosa*
A0102 Lea	I	*A. chinensis* var. *deliciosa*
A0186 Hayward clone 8	F	*A. chinensis* var. *deliciosa*
A0188 Summer 3373	F	*A. chinensis* var. *deliciosa*
A0041 Matua	M	*A. chinensis* var. *deliciosa*
A0042 Tomuri	M	*A. chinensis* var. *deliciosa*
A0105.2	F	*A. eriantha*
A0114.4	F	*A. eriantha*
A0114.2	M	*A. eriantha*
A0104.4	F	*A. chrysantha*
A0104.5	F	*A. chrysantha*
A0104.2	M	*A. chrysantha*
A0106.6	F	*A. latifolia*
A0162.5	M	*A. latifolia*
A0151.1	F	*A. macrosperma*
A0176	M	*A. macrosperma*
A0063	F	*A. kolomikta*
A0064	F	*A. kolomikta*
A0065	M	*A. kolomikta*
A0152	F	*A. arisanensis*
A0175	F	*A. valvata*
A0132.1	M	*A. valvata*
A083.3	M	*A. lanceolata*
A0074 Cornell	M	*A. arguta*
A0091 Issai	F	*A. arguta*
A0068	M	*A. arguta*
Miss green	F	*A. arguta*
A0103	M	*A. callosa*
A0124.1	M	*A. rufa*
A0124.2	F	*A. rufa*
A0121	M	*A. melanandra*
A0184	F	*A. melanandra*
A0180	F	*A. arguta* var. *purpurea*
A0069	F	*A. polygama*
A0070	M	*A. polygama*
A0050	F	*A. hemsleyana*

Column 1 = accession number or variety name; Column 2 = M=male, *F* = female, *I* = inconstant male; Column 3 = species and botanical variety.

Table 2a
PCR reaction set up using FrBy-UD-F/ FrBy-UD-R primers pair

Solution	Final concentration	Sample volume (μl)
Water		Variable
Buffer 10X	1X	1
FrBy-UD-F	0.2 μM	1
FrBy-UD-R	0.2 μM	1
dNTPs	0.2 mM	0.8
5' prime Taq Polymerase	2.5 U	0.1
Sample DNA	20 ng	
Total volume		10

Table 2b
PCR reaction set up using SyGI-selectF/ SyGI-selectR and Ank-UD-F/ Ank-UD-R primers pairs

Solution	Final concentration	Sample volume (μl)
Water		Variable
Buffer 10X	1X	1
SyGI-selectF	0.2 μM	1
SyGI-selectR	0.2 μM	1
Ank-UD-F	0.2 μM	1
Ank-UD-R	0.2 μM	1
dNTPs	0.2 mM	0.8
5' prime Taq Polymerase	2.5 U	0.1
Sample DNA	20 ng	
Total volume		10

Table 3a
PCR reaction set up using FrBy-UD-F/ FrBy-UD-R primers pair

Solution	Final concentration	Sample volume (μl)
Water		1
FrBy-UD-F	0.2 μM	1
FrBy-UD-R	0.2 μM	1
MyTaq Plant-Polymerase	1X	5
DNA dilution		2
Total volume		10

A dilution 2 : 38 was prepared from the crude extracts obtained from the quick extraction protocol. In this case, PCR reactions were performed using MyTaq^{TM} Plant-PCR kit (Bioline). The PCR reaction set up is reported in Tables 3a and 3b. Touch-down PCR cycling program was 95°C for 3 min, followed by 10 cycles at 95°C for 15 sec, 55°C (- 0.5°C at each cycle) for 15 sec and 72°C for 45 sec, and 20 cycles at 95°C for 15 sec, 50°C for 15 sec and 72°C for 45 sec, and a final extension at 72°C for 7 min.

The three gene targets were amplified with FAM or HEX dye-labelled forward primers. The PCR products were diluted 2 to 18 in case of *FrBy* amplification and 1 to 100 in case of *SyGI* and *Ank* amplification, mixed

Table 3b
PCR reaction set up using SyGI-selectF/ SyGI-selectR and Ank-UD-F/ Ank-UD-R primers pairs

Solution	Final concentration	Sample volume (μl)
Water		Variable
SyGI-selectF	0.2 μM	1
SyGI-selectR	0.2 μM	1
Ank-UD-F	0.2 μM	1
Ank-UD-R	0.2 μM	1
MyTaq Plant-Polymerase	1X	5
DNA dilution		2
Total volume		10

and run on a SeqStudio Genetic Analyzer (Thermo Fisher). The fragments were called and sized using the Microsatellite Analysis Software (MSA), which is a microsatellite genotyping module available on the Thermo Fisher Cloud.

3. Results and discussion

3.1. Method Set up

The amplification of *SyGI* gene, *Ank* gene and *FrBy* gene allowed the discrimination between male and female plants with a simple signal of presence/absence, both in the *Actinidia* species and in kiwifruit genotypes with different ploidy [21]. Specifically, male plants amplify three PCR products in contrast to female plants that amplify only the housekeeping gene (Fig. 1). The predicted sizes in *A. chinensis* genome were 90, 155, and 290 bp for *SyGI*, *Ank*, and *FrBy* markers, respectively.

The *SyGI* primer pair was published by Akagi and co-workers [18] and provides male-specific amplification in most of *Actinidia* species studied. *FrBy* and *Ank* primer pairs were designed specifically for this work. *Ank* marker acts as internal control to test successful PCR amplification, while our *FrBy* primers pair also face male-specific amplification and was used as double check of the discriminating test. PCR amplifications were initially set up using genomic DNA of different species of *Actinidia*, extracted using NucleoSpin® Plant II kit and visualized on agarose gel (Fig. 2). Later, we perfected a quick protocol that allowed the process to be speeded up consistently, allowing up to 192 samples to be screened in a working day.

3.2. Marker Assisted Selection (MSA) analysis for A. chinensis var. chinensis and A. chinensis var. deliciosa

We tested six different genotypes of *A. chinensis* var. *chinensis* and seven different genotypes of *A. chinensis* var. *deliciosa*: three male genotypes, two female genotypes and one inconstant male in the case of *A. chinensis* var. *chinensis*, and three male genotypes, three female genotypes and one inconstant –male in the case of *A. chinensis* var. *deliciosa*. Inconstant males were identified from our kiwifruit repository. These genotypes, also referred to as fruiting males, have bisexual flowers characterized by a small ovary with fewer carpels than a typical female vine, fewer ovules per carpel and shortened, thinner styles with small stigmata and they bear small fruits (20–40 g) with a few dozens of seeds. The ESD test has been effective in the discriminating between male and female for all genotypes.

Pherograms of female genotypes showed only one peak, as expected, corresponding to the fragment of 154 bp from the *Ank* gene (housekeeping gene) in both *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa* (Fig. 3). Pherograms of male and inconstant male genotypes showed three peaks, corresponding to the fragments 86 bp,

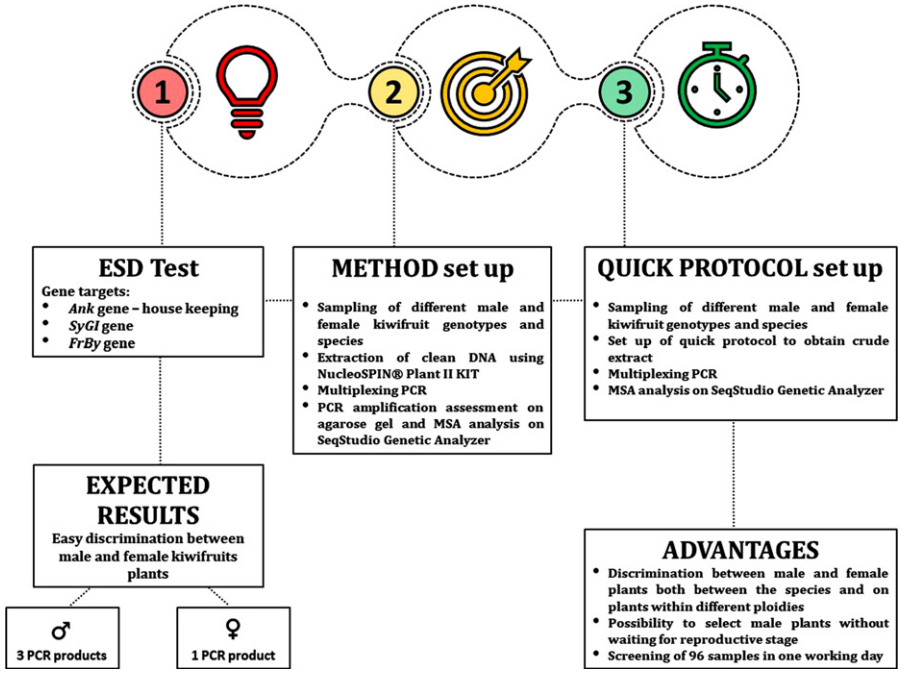

Fig. 1. Schematic representation of the ESD protocol.

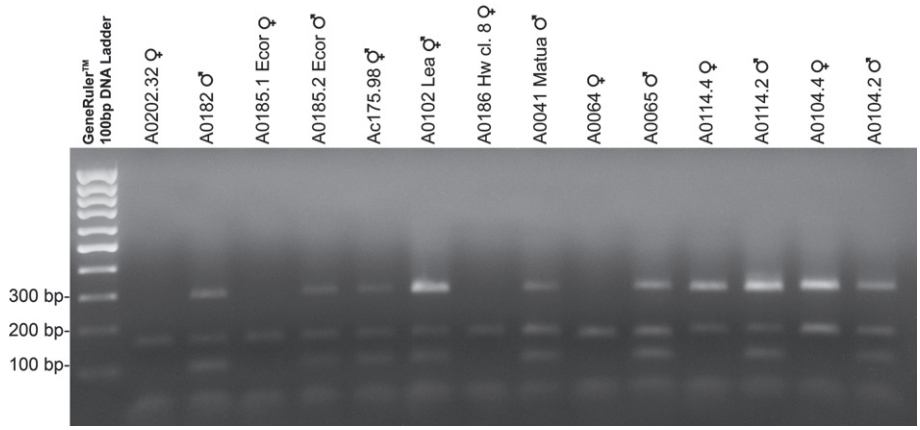

Fig. 2. Panel of amplifications for different genotypes and species of kiwifruit. The panel shows markers amplification in a sample of genotypes and species of kiwifruit. Genotypes were choose to show different case studies. More specifically the panel shows two diploid genotypes of *A. chinensis* var. *chinensis* (A0202.32 and A0182), two tetraploid genotypes of *A. chinensis* var. *deliciosa* (A0185.1 Ecor and A0185.2 Ecor), two inconstant male genotypes Ac175.98 (*A. chinensis* var. *chinensis*) and Ac102 Lea (*A. chinensis* var. *deliciosa*), two exaploid genotypes of *A. chinensis* var. *deliciosa* (A0186 Hw cl. 8 and A0041 Matua), two genotypes of *A. kolomikta* (A0064 and A0065), two genotypes of *A. eriantha* (A0114.4 and A0114.2) and two genotypes of *A. chrysantha* (A0104.4 and A0104.2). Male and inconstant male plants show three size-distinguishable PCR products: 90 bp, 154 bp and 290 bp respectively for *SyGI* marker, *Ank* marker and *FrBy* marker. The PCR products of the female plants were loaded into the wells 2, 4, 8, 10, 12, 14. In lanes 9 and 10, *A. kolomikta* female and male genotypes were loaded which showed a peak corresponding to a fragment 144 bp in size in the case of *Ank* marker (see text for details). GeneRulerTM 100 bp DNA Ladder (Thermo Fisher) was used for sizing the PCR products in the range of 100 to 1,000 bp on agarose gel.

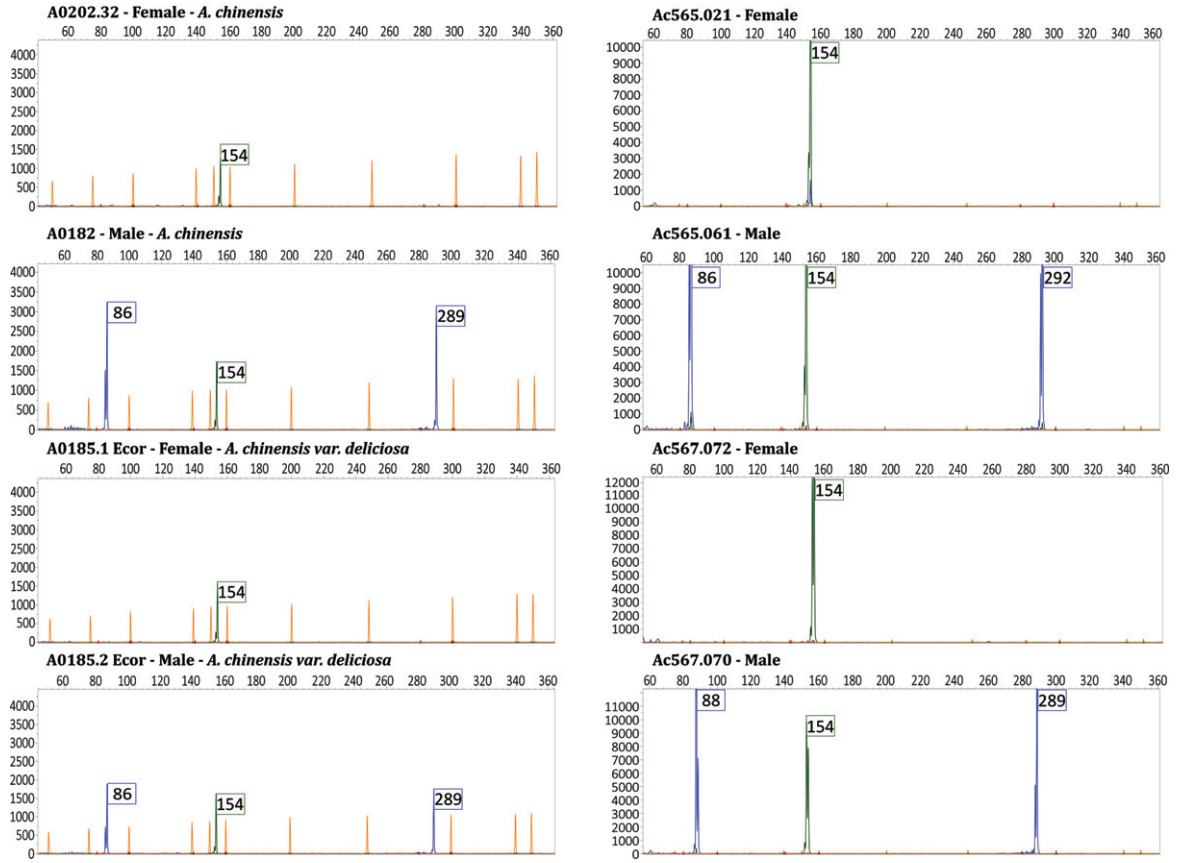

Fig. 3. Representative pherograms of eight genotypes analyzed. Pherograms of two individuals of *A. chinensis* var. *chinensis* and two individuals of *A. chinensis* var. *deliciosa*, female and male respectively on the left panel. Pherograms of two individuals of the cross-population Ac565 (a controlled cross *A. chinensis* var. *chinensis* 'Jintao' X Ac442 male pollen mix) and two individuals the cross-population Ac567 (a controlled cross *A. chinensis* var. *chinensis* 'Soreli' x *A. arguta* 'Cornell' male), female and male respectively on the right panel. Pherograms show the fragments size in base pairs (bp) on the x-axis and the intensity of the peaks on y-axis. The marker *Ank* (green peak) carries the fragment of 154 bp. The markers *SyGI* and *FrBy* (blue peaks) carry the fragment of 86 bp and 289 bp respectively in the case of *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa* genotypes. Fragment size for these two marker varies in the case of the cross populations. Male individuals of the cross-population Ac565 showed peaks corresponding to the fragments of 86 and 292 bp from the *SyGI* and *FrBy* genes respectively and fragments of 88 and 289 bp in the case of the cross population Ac567. In yellow the size-standard peaks.

154 bp and 289 bp, resulting from the amplification of *SyGI*, *Ank* and *FrBy* genes, respectively. Peak size was the same for all genotypes tested with the exception of the genotype Ac178.15. This male of *A. chinensis* shows a peak of 292 bp in the case of the *FrBy* gene. Results for all genotypes are summarized in Table 4. Our test could not distinguish between males and inconstant males.

3.3. MSA analysis of different Actinidia species

We tested 30 genotypes from the following species of *Actinidia*: *A. eriantha, A. chrysantha, A. latifolia, A. macrosperma, A. kolomikta, A. arisanensis* (the classification of this species is still uncertain), *A. valvata, A. lanceolata, A. arguta, A. callosa, A. rufa, A. melanandra, A. polygama* and *A. hemsleyana* (Table 1). We selected one female and one male genotype from each species when available. The housekeeping gene was amplified in

Table 4
Results of MSA analysis for *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa*

Genotype	Gender	Species	Marker Name	Gel electrophoresis	Dye Color	Size
A0181	M	*A. chinensis* var. *chinensis*	Ank	yes	Green	NA
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
A0202.32	F	*A. chinensis* var. *chinensis*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0182	M	*A. chinensis* var. *chinensis*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
Ac0453.004	F	*A. chinensis* var. *chinensis*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
Ac0178.15	M	*A. chinensis* var. *chinensis*	Ank	yes	Green	154
			FrBy	yes	Blue	292
			SyGI	yes	Blue	86
Ac0275.98	I	*A. chinensis* var. *chinensis*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
A0185.1 Ecor	F	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0185.2 Ecor	M	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
A0102 Lea	I	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
A0186 Hayward clone 8	F	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0188 Summer 3373	F	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0041 Matua	M	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
A0042 Tomuri	M	*A. chinensis* var. *deliciosa*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86

Column 1 = accession number or variety name; Column 2 = M = male, *F* = female, *I* = inconstant male; Column 3 = species and botanical variety; Column 4 = marker name as specified in the text; Column 5 = amplicons separated on gel electrophoresis (yes = band detected, no = band not detected); Column 6 = dye colors of the peaks after capillary electrophoresis analysis; Column 7 = Size of the peaks in base pairs (NA = PCR amplification failed; – = PCR amplification not expected).

Table 5
Results of MSA analysis of different *Actinidia* species

Genotype	Gender	Species	Marker Name	Gel electrophoresis	Dye Color	Size
A0105.2	F	*A. eriantha*	Ank	yes	Green	154
			FrBy	yes	Blue	*282**
			SyGI	no	Blue	–
A0114.4	F	*A. eriantha*	Ank	yes	Green	154
			FrBy	yes	Blue	*282**
			SyGI	no	Blue	–
A0114.2	M	*A. eriantha*	Ank	yes	Green	154
			FrBy	yes	Blue	282
			SyGI	yes	Blue	88
A0104.4	F	*A. chrysantha*	Ank	yes	Green	154
			FrBy	yes	Blue	*289**
			SyGI	no	Blue	–
A0104.5	F	*A. chrysantha*	Ank	yes	Green	154
			FrBy	yes	Blue	*289**
			SyGI	no	Blue	–
A0104.2	M	*A. chrysantha*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	89
A0152	F	*A. arisanensis*	Ank	yes	Green	154
			FrBy	no	Blue	*289**
			SyGI	no	Blue	–
A0106.6	F	*A. latifolia*	Ank	yes	Green	154
			FrBy	yes	Blue	*289**
			SyGI	no	Blue	–
A0162.5	M	*A. latifolia*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	no	Blue	NA
A0151.1	F	*A. macrosperma*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0183	M	*A. macrosperma*	Ank	yes	Green	154
			FrBy	yes	Blue	292
			SyGI	yes	Blue	89
A0063	F	*A. kolomikta*	Ank	yes	Green	144
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0064	F	*A. kolomikta*	Ank	yes	Green	144
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0065	M	*A. kolomikta*	Ank	yes	Green	144
			FrBy	yes	Blue	289
			SyGI	yes	Blue	88

(Continued)

Table 5
(Continued)

Genotype	Gender	Species	Marker Name	Gel electrophoresis	Dye Color	Size
A0175	F	*A. valvata*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0132.1	M	*A. valvata*	Ank	yes	Green	154
			FrBy	yes	Blue	292
			SyGI	yes	Blue	89
A0083.3	M	*A. lanceolata*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	89
A0074 Cornell	M	*A. arguta*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	88
A0091 Issai	F	*A. arguta*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0068	M	*A. arguta*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	88
Miss green	F	*A. arguta*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0103	M	*A. callosa*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	no	Blue	88
A0124.1	M	*A. rufa*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	86
A0124.2	F	*A. rufa*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0121	M	*A. melanandra*	Ank	yes	Green	154
			FrBy	yes	Blue	289
			SyGI	yes	Blue	88
A0184	F	*A. melanandra*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0180	F	*A. arguta* var. *purpurea*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–
A0069	F	*A. polygama*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–

(Continued)

Table 5
(Continued)

Genotype	Gender	Species	Marker Name	Gel electrophoresis	Dye Color	Size
A0070	M	*A. polygama*	Ank	yes	Green	154
			FrBy	yes	Blue	292
			SyGI	yes	Blue	91
A0050	F	*A. hemsleyana*	Ank	yes	Green	154
			FrBy	no	Blue	–
			SyGI	no	Blue	–

Column 1 = accession number or variety name; Column 2 = M=male, *F* = female; Column 3 = species and botanical variety; Column 4 = marker name as specified in the text; Column 5 = amplicons separated on gel electrophoresis (yes = band detected, no = band not detected); Column 6 = dye colors of the peaks after capillary electrophoresis analysis; Column 7 = Size of the peaks in base pairs (NA = PCR amplification failed; – = PCR amplification not expected; *size of the peaks in base pairs reported in *italics* = unexpected amplification of the *FrBy* marker in female genotypes).

Fig. 4. Pollen fertility controlled by *FrBy* gene. A comparison between *A. eriantha* selfed and open pollinated: fruit set failed after self-pollination of bag-isolated flowers on the left, while open-pollinated fruits are developing normally on the right. See text for details.

both male and female genotypes and pherograms showed a peak corresponding to the fragment 154 bp in size for all *Actinidia* species with the exception of *A. kolomikta* which showed a peak corresponding to a fragment of 144 bp in size in both the two female genotypes and in the male one. This deletion can be detected also by gel electrophoresis (Fig. 2). Pherograms of all males in different species showed three peaks resulting from the *SyGI*, *Ank* and *FrBy* markers amplification respectively. *SyGI* amplification failed only in *A. latifolia*, presumably because of a mutation in the annealing site of the primers. *SyGI* amplicon size for the different species ranges from 86 bp of *A. rufa*, to 91 bp of *A. polygama*. The amplification of *FrBy* resulted in the amplification of a 289 bp peak in all species except *A. macrosperma*, *A. valvata* and *A. polygama*, where the fragment size was of 292 bp and *A. eriantha* where the fragment was of 282 bp. Specific *SyGI*, *FrBy* and *Ank* genes amplicon sizes of all species are summarized in Table 5.

Table 6
Results of MSA analysis for the cross populations Ac565 and Ac567

Genotype	Gender	Marker Name	Dye Color	Size
Ac565.008	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.020	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.021	F	Ank	Green	154
		FrBy	Blue	–
		SyGI	Blue	–
Ac565.024	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.025	F	Ank	Green	154
		FrBy	Blue	–
		SyGI	Blue	–
Ac565.033	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.035	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.042	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.044	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.057	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.058	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.059	F	Ank	Green	154
		FrBy	Blue	–
		SyGI	Blue	–
Ac565.061	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.066	F	Ank	Green	154
		FrBy	Blue	–
		SyGI	Blue	–

(Continued)

Table 6
(Continued)

Genotype	Gender	Marker Name	Dye Color	Size
Ac565.068	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.071	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.074	F	Ank	Green	154
		FrBy	Blue	–
		SyGI	Blue	–
Ac565.077	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.084	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac565.089	M	Ank	Green	154
		FrBy	Blue	292
		SyGI	Blue	86
Ac567.030	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.042	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.048	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.059	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.061	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.066	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.070	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.072	F	Ank	Green	154
		FrBy	Blue	–
		SyGI	Blue	–

(Continued)

Table 6
(Continued)

Genotype	Gender	Marker Name	Dye Color	Size
Ac567.078	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.096	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88
Ac567.127	M	Ank	Green	154
		FrBy	Blue	289
		SyGI	Blue	88

Column 1 = accession number; Column 2 = M=male, *F* = female; Column 3 = marker name as specified in the text; Column 4 = dye colors of the peaks after capillary electrophoresis analysis; Column 5 = Size of the peaks in base pairs (– = PCR amplification not expected).

In four specific cases of female genotypes of *A. eriantha*, *A. chrysantha*, *A. latifolia* and *A. arisanensis* we observed the unexpected amplification of the *FrBy* marker. Female genotypes of *A. eriantha* amplified a fragment of 282 bp in size, while the remaining three female genotypes amplified a fragment of 289 bp in size. At flowering, flowers of *A. eriantha* were bagged to avoid external pollen contamination. In these conditions, we tested pollen fertility controlled by *FrBy* gene and if gene function was not impaired, we expected to observe evidence of self-pollination. We did not observe self-pollination and fruit set (Fig. 4). Moreover, we amplified the entire *FrBy* gene using primers pair described by Akagi and co-workers [19] producing an amplicon of 723 bp, but in the case of *A. eriantha* female the amplification did not occur. These observations supported the speculation that at least four A*ctinidia* species traces of an interrupted or degenerate *FrBy* gene sequence are still present, but further investigations are needed.

3.4. MSA analysis of cross-populations

We tested 20 individuals collected from the cross-population named Ac565 (a controlled cross *A. chinensis* var. *chinensis* 'Jintao' X Ac442 male pollen mix) and 11 individuals from Ac567 (a controlled cross *A. chinensis* var. *chinensi*s 'Soreli' x *A. arguta* 'Cornell' male). Phenotypic evaluation identified 15 male and five female plants in the Ac565 cross population and ten male and one female plants in the Ac567 cross population. The ESD test was effective in discriminating male and female individuals. Pherograms of female genotypes showed only one peak corresponding to the 154 bp fragment from the *Ank* gene (housekeeping gene) for both cross populations. Pherograms of male individuals showed three peaks corresponding to the fragments of 86, 154, and 292 bp resulting from the *SyGI*, *Ank* and *FrBy* genes amplification respectively in the case of the cross population Ac565 and fragments of 88, 154, and 289 bp in the case of the cross population Ac567 (Fig. 3). Results for all the genotypes are summarized in Table 6.

4. Conclusion

Methods for determining sex are valuable in dioecious fruit crops because plants cannot be predicted morphologically at an early seedling stage. Prediction of sex at the seedling stage would save a huge amount of resources, such as space required for planting, and the labor of plant management.

DNA based markers for sex-determinants have proved to be a very successful tool for inferring the gender in dioecious plants. Sex-linked molecular markers have been already developed for different woody plants such as papaya (*Carica papaya*) [22], ginkgo (*Ginkgo biloba*) [23], date-plum (*Diospyros lotus*) [24], mulberry (*Morus alba* L.) [25], *Garcinia subelliptica* Merr. [26], pistachio (*Pistacia vera*) [27]. Recently, Chłosta and co-workers [28] exploited the *SyGI* marker to exclude male and identify female lines in endosperm-derived kiwifruit callus and its regenerants.

In our work, lack of amplification of the *SyGI* and *FrBy* markers allows the selection of fruit-bearing females in almost all the genotypes tested. Few exceptions were identified: in female genotypes of *A. eriantha*, *A. chrysantha*, *A. latifolia* and *A. arisanensis* we observed an unexpected amplification of the *FrBy* gene, but we ruled out the potential presence of an intact and functional copy of the gene in one of these species. However, with the exception of *A. latifolia* that did not amplify the *SyGI* marker probably because a specific mutation in annealing site, for the others 14 species tested the *SyGI* marker allows the discrimination of male plants from female ones. We did not develop a specific test for *A. latifolia* with a dedicated primer for gender selection. In conclusion, we demonstrate that the ESD test is effective and reliable in sex discrimination for kiwifruit and several other A*ctinidia* species. The test is an important tool because could help in breeding programs. Moreover, the possibility of exploiting a quick protocol of extraction would greatly reduce the time needed for the screening of a large number of plants. We could screen up to 192 samples in a working day using a low processivity platform such as the SeqStudio Genetic Analyzer (Thermo Fisher).

Author contributions

GC conceived and planned the research; GDM did the experimental work in the laboratory and analysis; GDM and GC wrote the first draft of the text; RT revised the first draft; all authors contributed to the final version of the manuscript.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

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