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Paternity analysis in a 'Rainier' open pollination population using *S*-alleles and microsatellite genotyping

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Abstract

Fruit breeding programs usually use controlled hand pollination among cultivars and advanced selections for obtaining segregating populations to select new cultivars. In sweet cherries, however, sometimes in controlled pollination few hybrids are obtained. Caging whole trees with bees and flowers of the pollinating cultivar is sometimes used to obtain larger hybrid populations. To generate large segregating populations for the Chilean Sweet Cherry Breeding Program (run by the Consortium of BioFrutales S.A. and Instituto de Investigaciones Agropecuarias), the initial strategy was to harvest seeds from open pollinated self-incompatible cultivars maintained at the germplasm collection orchard of Univiveros, one of the leading fruit tree nurseries in Chile. While the female parent is known, the male parent is unknown. The pollen source will depend on the cultivars present in the area, the flowering time and the compatibility of the cultivars involved. In order to identify pollinators of the self-incompatible 'Rainier', which is surrounded by several other cultivars in the collection, a group of them were proposed as the most possible pollen donors considering their flowering time and location within the orchard. An initial screening using *S*-allele genotyping of 675 one-year-old seedlings from 'Rainier' open-pollination allowed for the identification of several *S*-genotypes, which suggested putative pollinators. These analyses identified two main putative pollen donors: 'Rivedel' (232 individuals, 34% of total) and 'Vanda' (101 individuals, 15% of the total). The paternity of these cultivars was confirmed or rejected after additional analysis with at least five highly informative microsatellite markers. The segregating population resulting from the cross 'Rainier' × 'Rivedel' (*n*=232) is currently being analyzed with microsatellite markers in order to build a linkage map for sweet cherry. In this population, parents exhibit contrasting phenotypes for skin and flesh color and berry firmness, among other agronomically important traits, making this population very interesting for future genetic studies.

Keywords: breeding program, cultivars, pollinator, molecular markers, *Prunus avium* L.

INTRODUCTION

All sweet cherry cultivars grown in Chile are from foreign origin and despite most of them exhibiting good fruit quality, at the same time they show poor storage capacity and low ability to travel long distances. This is a mayor constraint for the Chilean sweet cherry industry, the biggest in the Southern Hemisphere. To overcome some of the shortcomings of the foreign cultivars, in 2009 BioFrutales S.A., a consortium of nurseries, research institutions and universities started the first sweet cherry breeding program in Chile.

The traditional breeding strategy in cherries has been the selection of superior phenotypes in segregating populations obtained after controlled hand pollinations among cultivars and advanced selections. One disadvantage of this method is that few hybrids are usually obtained. Another strategy for generating larger segregating populations at the Chilean Program was to collect seeds from open pollinated self-incompatible cultivars.

Self-incompatibility in sweet cherry is controlled by the multiallelic *S*-locus (Crane and

Lawrence, 1928) and cultivars can be categorized in various self-incompatibility groups (Tobutt et al., 2004; Schuster et al., 2007). The *S*-locus includes two genes: *S-RNase*, the style determinant, which encodes for a protein with ribonuclease activity (Bošković and Tobutt, 1996; Tao et al., 1997, 1999; Ushijima et al., 1998) and *SFB*, the pollen determinant, which encodes for a protein with an F-box motif (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003). The *S-RNase* gene has two introns and their amplification by the polymerase chain reaction (PCR) has been successfully used to detect different *S*-alleles in sweet cherry genotypes (Tao et al., 1999; Sonneveld et al., 2001, 2003; Wiersma et al., 2001; Wünsch and Hormaza, 2004; De Cuyper et al., 2005; Schuster et al., 2007; Gisbert et al., 2008; Szikriszt et al., 2013).

Microsatellite (SSR) markers have been used for cultivar identification, genetic diversity studies, construction of linkage maps and paternity tests (Zane et al., 2002; Kalia et al., 2011). Currently, they are the molecular markers of choice for many types of studies given their high polymorphism and because they are co-dominant, allowing identifying both alleles in diploid organisms (Parida et al., 2009; Kalia et al., 2011).

In this paper, we used *S*-allele typing coupled with SSR genotyping to identify the putative male parental genotype of a population from 'Rainier' sweet cherry open-pollination.

MATERIALS AND METHODS

A total of 675 one-year-old seedlings were used in this study. Seedlings were obtained by collecting seeds from 'Rainier' fruits. This tree is surrounded by 'Rivedel' (S_1S_9), 'Vanda' (S_1S_6), 'Van' (S_1S_3), 'Bing' (S_3S_4) and 'Lapins' (S_1S_4) among other cultivars planted in the germplasm collection of Univiveros. This fruit tree nursery is located in Paine, close to Santiago, in Chile. These cultivars were selected as putative parents among 54 other cultivars present at the collection because of their blooming date and location in the orchard. For DNA extraction, leave samples were collected, transported to the laboratory in refrigerated containers and immediately stored at -80°C for later use. DNA was extracted following the method described by Lodhi et al. (1994). DNA concentration was measured using an Infinite® 200 PRO NanoQuant microplate reader (Tecan Tradind AG, Männedorf, Switzerland).

For *S*-allele typing, samples were analysed with the consensus primers PaConsII for amplification of the second intron of *S-RNase* gene (Sonneveld et al., 2003). Approximately 20-80 ng of genomic DNA was used for PCR. Amplification and detection of PCR products was done following the protocol described by Sonneveld et al. (2003). For SSR analyses, samples were PCR-amplified using seven pairs of primers: BPPCT-026 and BPPCT-038 (Dirlewanger et al., 2002); PMS-30 (Struss et al., 2002); PMS-67 (Cantini et al., 2001); UCD-CH11, UCD-CH12 and UCD-CH26 (Struss et al., 2003). PCR reactions were carried out in a total volume of 12 µL, with 20 ng genomic DNA, 0.5 µM of each reverse and forward primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 2.4 µl Colorless GoTaq® Reaction Buffer (5×), and 0.25 U GoTaq® DNA polymerase (Promega, Madison, WI). PCR reactions were carried out on an XP Cycler thermocycler (Bioer Technology, Hi-tech (Binjiang) District Hangzhou, China). PCR temperature profile, electrophoresis and silver-staining were done according to Rojas et al. (2008).

RESULTS AND DISCUSSION

Because of blooming date and location in the orchard, we were able to propose five probable male parents for the segregating open pollinated 'Rainier' population. These cultivars were 'Rivedel' (S_1S_9), 'Vanda' (S_1S_6), 'Van' (S_1S_3), 'Bing' (S_3S_4) and 'Lapins' (S_1S_4). All these putative male parents have red-dark to red skin and flesh. DNA from these cultivars plus maternal DNA ('Rainier', S_1S_4) were used as references for the screening of 675 seedlings by *S*-allele typing. Several band patterns were identified recurrently, suggesting that male parents could be few, and that the number of individuals of the same cross could be large. An example of PCR banding patterns after amplification with consensus primers PaConsII is shown in Figure 1. The analysis of the amplification profiles of all the seedlings

showed two main groups, corresponding to almost 50% of the population: individuals with the *S*-alleles genotypes S_1S_9 and S_4S_9 (232 individuals, 34% of total) and individuals with the *S*-alleles genotypes S_1S_6 and S_4S_6 (101 individuals, 15% of the total). These allelic combinations could come from 'Rivedel' and 'Vanda', respectively. These paternities were confirmed or rejected after the analysis with seven SSR markers (Figure 2). The SSR markers were previously selected as the most polymorphic and discriminant among sweet cherry cultivars (unpublished results). For 'Rivedel', 218 from 232 individual (94%) were confirmed as coming from crossing 'Rainier' × 'Rivedel', and for 'Vanda' only partial results are available, with 50 from 101 individuals confirmed with four SSRs. The genetic characterization of the remaining plants of the population will be analyzed using the same markers system.

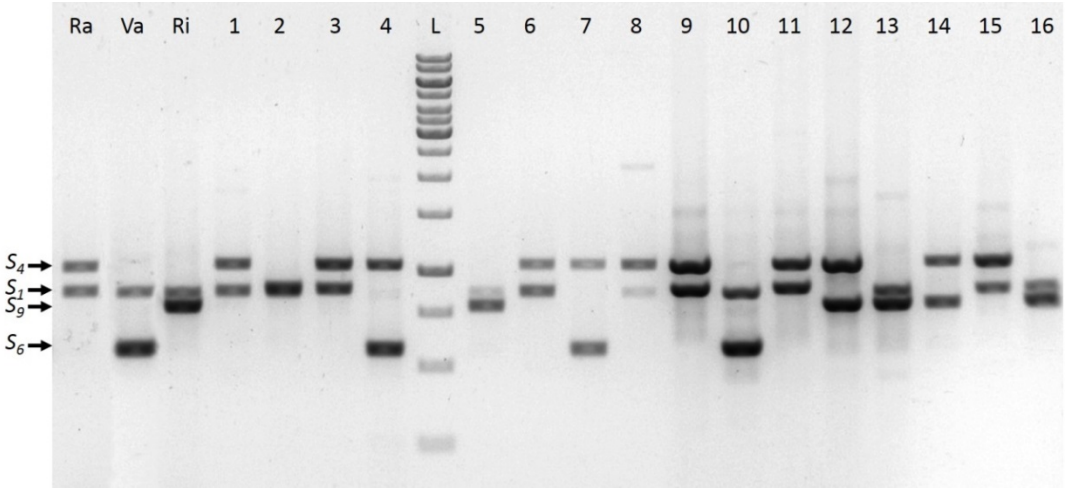

Figure 1. Agarose gel electrophoresis of *S*-alleles using consensus primers PaConsII, for amplification of the second intron of the *S-RNase* gene (Sonneveld et al., 2003) of 'Rainier' (Ra, S_1S_4), 'Vanda' (Va, S_1S_6), 'Rivedel' (Ri, S_1S_9) and 16 seedlings. 'Vanda' could be pollen donor for individuals 4, 7 and 10. 'Rivedel' could be pollen donor for individuals 5, 12, 13, 14 and 16. Other individuals could have other pollen donors present in the orchard. Alleles size: S_1: 874 bp; S_4: 1064 bp; S_6: 577 bp; S_9: 798 bp. L: Thermo Scientific GeneRuler™ 1 kb DNA Ladder.

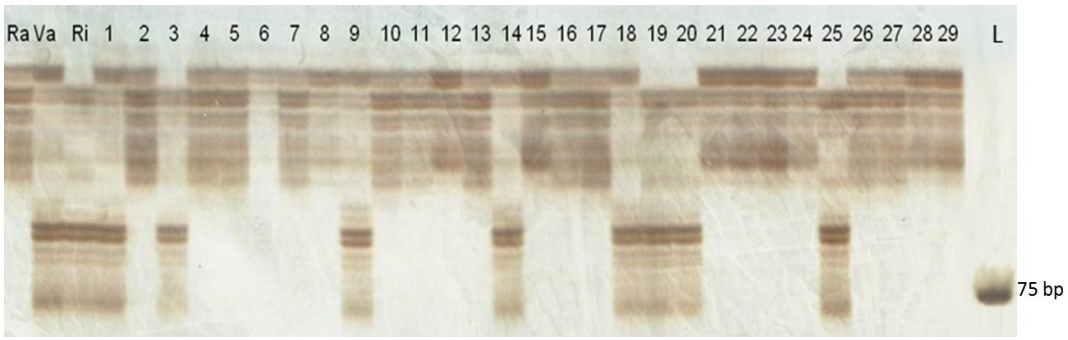

Figure 2. Polyacrilamide gel electrophoresis patterns of microsatellite amplification (UCD-CH39 marker) for 'Rainier' (Ra), 'Vanda' (Va), 'Rivedel' (Ri) and 29 seedlings coming from 'Rainier' × 'Rivedel' and 'Rainier' × 'Vanda' crossings. L: Promega 25 bp DNA Step Ladder.

Breeders often use hand pollination of emasculated flowers for hybridization to avoid self-pollination and to make flowers unattractive to pollinator insects (Free, 1964), thus increasing the chances of ovule fertilization by the desired pollen (Arbeloa et al., 2006).

However, flower emasculation may cause premature ovule degeneration, preventing fruit set, as has been reported in Japanese plums (Guerra et al., 2010). Emasculation also affects pollen tube growth and directionality in the ovary, thus minimizing fertilization (Hedhly et al., 2009); indeed, emasculation is the most probable cause of the erratic results obtained after artificial pollination from year to year. Caging whole trees with bees plus flowers of the pollinating cultivar has also been used to increase fruit set, but even though fruit set is effectively improved, it is not as much as collecting seeds from open pollinated plants. However, collecting seed from an open pollinated self-incompatible parent is not a universal solution to obtain large segregating populations, because crossing ability is dependent not only of the incompatibility group, but also on the time of flowering. In this particular case, we selected 'Rainier' as the female parent because of its fruit quality and consumer acceptability, expecting that 'Rivedel' and 'Vanda' could contribute to improve the postharvest life, firmness and skin and flesh colour of the segregants. A segregating population in the order of 500 seedlings is the goal of the Breeding Program for each cross. These segregating populations can be used not only for selection of superior genotypes, but also for mapping and QTL identification of traits of interest.

CONCLUSIONS

The use of *S*-genotyping coupled with SSR analysis is a reliable tool for the identification of putative pollen donor in open pollinated segregating populations derived from self-incompatible sweet cherry parents.

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