Use of *S*-alleles and microsatellite genotyping for parental identification and to verify interspecific hybridization in a Chilean *Prunus* rootstock breeding program

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Abstract

The use of molecular markers to complement phenotypic selection has potential for making both scion and rootstock breeding programs more efficient. Additionally, molecular markers can be used for cultivar identification and to test the hybrid nature of the segregants. In Chile, the Centro de Estudios Avanzados en Fruticultura (CEAF), started a breeding program for *Prunus* rootstocks where a series of interspecific crosses are underway, based on a *Prunus* germplasm collection available at this center. Molecular markers were used for cultivar identification and for determining the hybrid nature of individuals coming from interspecific crosses. For cultivar identification, *S*-allele genotyping was used to characterize 23 *Prunus* rootstocks. Preliminarily, these results allowed the differentiation between genotypes coming from seed propagation from those clonally propagated. To test for true hybrids arising from interspecific crosses, *S*-allele genotyping were used together with microsatellite markers. A population of about 100 seedlings, arising from the cross of *P. mahaleb* × *P. avium*, both diploid species, turned out to be selfs of *P. mahaleb*. A second population of 26 seedlings obtained from the cross of *P. avium* (2*n*=16) × *P. cerasus* (4*n*=32), resulted, as expected, in triploid hybrids (3*n*=24). These results are useful both to make the program more cost-effective and to allow for better planning of the interspecific crosses.

Keywords: molecular markers, germplasm, cherry rootstocks

INTRODUCTION

Chile has the leading fruit industry in the Southern Hemisphere (Food and Agriculture Organization of the United Nations, 2006), with stone fruits (peach, plum, prune, apricot, almond and cherry) being the 4th group of importance, following grapes, pome fruits, and avocados. Most of the cultivars grown in Chile are of foreign origin, despite the existence of breeding programs for peaches and sweet cherries, the first of which has just released their first cultivar (Infante et al., 2011). The Centro de Estudios Avanzados en Fruticultura (CEAF) recently started the only rootstock breeding program for *Prunus* rootstocks in Chile, where a series of interspecific crosses are underway based on a *Prunus* germplasm collection existing at the Center. The strategy of this Program is to incorporate the use of molecular markers both for cultivar identification and for the determination of the hybrid nature of individuals coming from interspecific crosses. Also, the use of molecular markers to complement the phenotypic selection has the potential to increase the efficiency of both scion and rootstock breeding programs. Marker-assisted selection can shorten the time required to obtain new cultivars and can make the process more cost-effective than selection based exclusively on phenotype, because useless material can be discarded at the seedling stage (Kalia et al.,

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2011; Parida et al., 2009).

In this paper we present the use of *S*-allele amplification, which is the result of PCR amplification of a hypervariable region from *S-RNase* gene, the female determinant of gametophytic self-incompatibility in *Rosaceae* (Bošković and Tobutt, 1996; Tao et al., 1997, 1999; Ushijima et al., 1998). *S*-alleles amplification coupled with microsatellites genotyping was used for identification of *Prunus* rootstock cultivars and to test the hybrid nature of the progenies.

MATERIALS AND METHODS

Plant material

Plant material comes from the *Prunus* germplasm collection of CEAF, where 23 plants 2-5 years old exist (Table 1). An additional 100 seedlings arising from a cross of *P. mahaleb* 'Pontaleb' × *P. avium* 'Bing', both diploid species, and 26 2-month-old seedlings coming from the cross of *P. avium* 'Bing' (2*n*=16) × *P. cerasus* 'Cab 6P' (4*n*=32), came from tissue culture after in vitro embryo rescue.

Table 1. *Prunus* rootstocks available at CEAF germplasm collection. Rootstock and parental ploidy are shown in parentheses.

Rootstock	Parents (genetic background)
Adara (2*n*)	*P. cerasifera* (2*n*)
Adesoto 101 (6*n*)	*P. insititia* (6*n*)
Cab 6P (4*n*)	*P. cerasus* (4*n*)
Citation (2*n*)	*P. salicina* (2*n*) × *P. persica* (2*n*)
Colt (3*n*)	*P. avium* (2*n*) × *P. pseudocerasus* (4*n*)
Gisela 5 (3*n*)	*P. cerasus* (4*n*) × *P. canescens* (2*n*)
Gisela 6 (3*n*)	*P. cerasus* (4*n*) × *P. canescens* (2*n*)
Gisela 12 (3*n*)	*P. cerasus* (4*n*) × *P. canescens* (2*n*)
GxN (Garnem) (2*n*)	*P. persica* (2*n*) × *P. dulcis* (2*n*)
Ishtara (2*n*)	[*P. cerasifera* (2*n*) × *P. salicina* (2*n*)] × [(*P. cerasifera* (2*n*) × *P. persica* (2*n*)]
Mariana 2624 (2*n*)	*P. cerasifera* (2*n*) × *P. munsoniana* (2*n*)
Maxma 14 (2*n*)	*P. mahaleb* (2*n*) × *P. avium* (2*n*)
Maxma 60 (2*n*)	*P. mahaleb* (2*n*) × *P. avium* (2*n*)
Mazzard F12/1 (2*n*)	*P. avium* (2*n*)
Mericier (2*n*)	*P. avium* (2*n*)
Myrobalan (2*n*)	*P. cerasifera* (2*n*)
Nemaguard (2*n*)	*P. persica* (2*n*) × *P. davidiana* (2*n*)
Pacer 00-05 (4*n*)	[*P. pumila* (2*n*) × *P. armeniaca* (2*n*)] × (*P. domestica*) (6*n*)
Pomona (2*n*)	*P. persica* (2*n*)
Pontaleb (2*n*)	*P. mahaleb* (2*n*)
Rootpac 40 (2*n*)	[*P. dulcis* (2*n*) × *P. persica* (2*n*)] × [(*P. dulcis* (2*n*) × *P. persica* (2*n*)]
Rootpac 70 (2*n*)	[*P. persica* (2*n*) × *P. davidiana* (2*n*)] × [(*P. dulcis* (2*n*) × *P. persica* (2*n*)]
Rootpac 90 (2*n*)	[*P. persica* (2*n*) × *P. davidiana* (2*n*)] × [(*P. dulcis* (2*n*) × *P. persica* (2*n*)]

Methods

For DNA extraction, young leaf 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).

The *S*-haplotype profile for each sample was obtained using the consensus primers PaConsII for amplification of the second intron of the *S*-RNase gene (Sonneveld et al., 2003), which is involved in self-incompatibility (De Nettancourt, 2001). Approximately 20-80 ng of genomic DNA was used for PCR. Amplification and detection of PCR products followed the protocols of Sonneveld et al. (2003).

Samples were PCR-amplified using 4 microsatellite markers: BPPCT-026 and BPPCT-037 (Dirlewanger et al., 2002), PMS-67 (Cantini et al., 2001) and UCD-CH18 (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, USA). PCR reactions were carried out on a XP Cycler thermocycler (Bioer Technology, Hi-tech (Binjiang) District Hangzhou, China). PCR temperature profile, electrophoresis and silver-stain were done following the methods described by Rojas et al. (2008).

RESULTS AND DISCUSSION

Two types of molecular markers were used to analyze the individual plants of the germplasm collection and to confirm the clonal nature of the propagated rootstocks. First, we used the amplification of a hypervariable region of the *S*-RNase gene, which is involved in the gametophytic self-incompatibility system present in the *Rosaceae* family (De Nettancourt, 2001). Self-incompatibility has been extensively studied at the molecular level, and it is now known that this character is controlled by a single locus with multiple alleles (Tao and Iezzoni, 2010). Conserved primers for *S*-allele amplification of the 23 *Prunus* rootstocks were used (Table 1). Figure 1a shows the *S*-allele profile for each rootstock, with sizes ranging from 500 to 3,000 bp. There are a high number of alleles in this rootstock group, but there is not information available on the sequence of each allele. Microsatellites were also used, as shown on Figure 1b. Microsatellites have been used for cultivar identity confirmation, genetic diversity studies, construction of linkage maps and paternity tests, among other uses (Zane et al., 2002; Kalia et al., 2011). Results obtained with both kinds of markers allowed for the differentiation of segregants coming from seed propagation ('Citation', 'Myrobalan' and 'Pontaleb') from those coming from clonal propagation.

The same strategy of *S*-allele and microsatellite amplification was used to test for the hybrid nature of segregants coming from interspecific crosses. Fifty seedlings coming from the cross of 'Pontaleb' × 'Bing' were used to test for hybridity. Figure 2a shows the allelic pattern observed in the 50 seedlings, showing that all segregants turned out to be a selfing of 'Pontaleb', since the amplification showed only 'Pontaleb' alleles. This result was sufficient to assess the selfing nature of the seedlings and there was no need of further microsatellite studies. On the other hand, results from *S*-alleles and microsatellite amplification for a population of 26 seedlings coming from a cross of 'Bing' × 'Cab 6P', indicated that all individuals were triploid (3*n*=24), confirming the interspecific origin of the hybrids (Figure 2b, c).

Genetic resource characterization, management and conservation are essential for breeding programs. Bassil and Lewers (2009) reported the use of molecular markers in the *Rosaceae* family by breeders throughout the world. Molecular characterization of *Prunus* rootstocks is useful for the proper identification of the propagation material, and to overcome the environmental × genotypic interaction during evaluation. The analyses of genetic diversity among groups of *Prunus* rootstocks using cross-transferable microsatellite markers (markers designed in a species that can be utilized for analysis of other species) have been reported (Serrano et al., 2002; Liu et al., 2007; Bouhadida et al., 2009; Arismendi et al., 2012). Also, there is little information on the genetics of self-incompatibility in *Prunus* rootstocks, where a large number of cultivars come either from natural or artificial interspecific hybridization. We used the *S*-allele amplification to confirm the hybrid nature of the segregants, but the technique could be also useful to investigate the incompatibility groups in *Prunus* rootstocks, which may help to predict the crossability among species, since this is one of the causes of the failure of interspecific crosses.

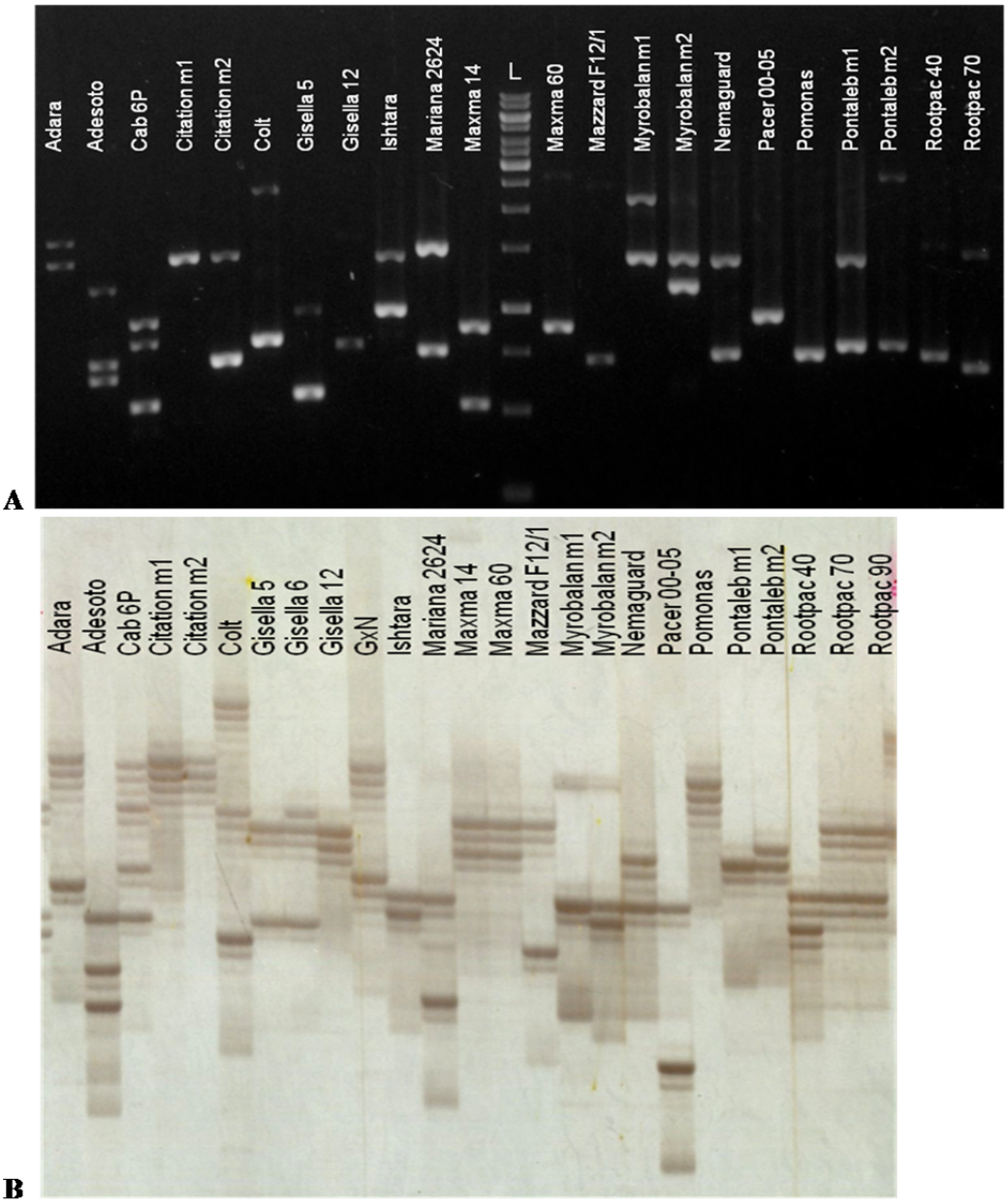

Figure 1. *S*-alleles and microsatellite profiling of *Prunus* rootstocks. (A) PCR analysis of the genotype of different *Prunus* rootstocks amplified with consensus primers for *S*-RNase gene. (B) Example of microsatellite profile using PMS-67, a microsatellite marker. One sample of each rootstock is shown, except for 'Citation', 'Myrobalan' and ´Pontaleb', which proceed from seed propagation. L: GeneRuler™ 1 kb DNA Ladder (Thermo Fischer Scientific, West Palm Beach, FL).

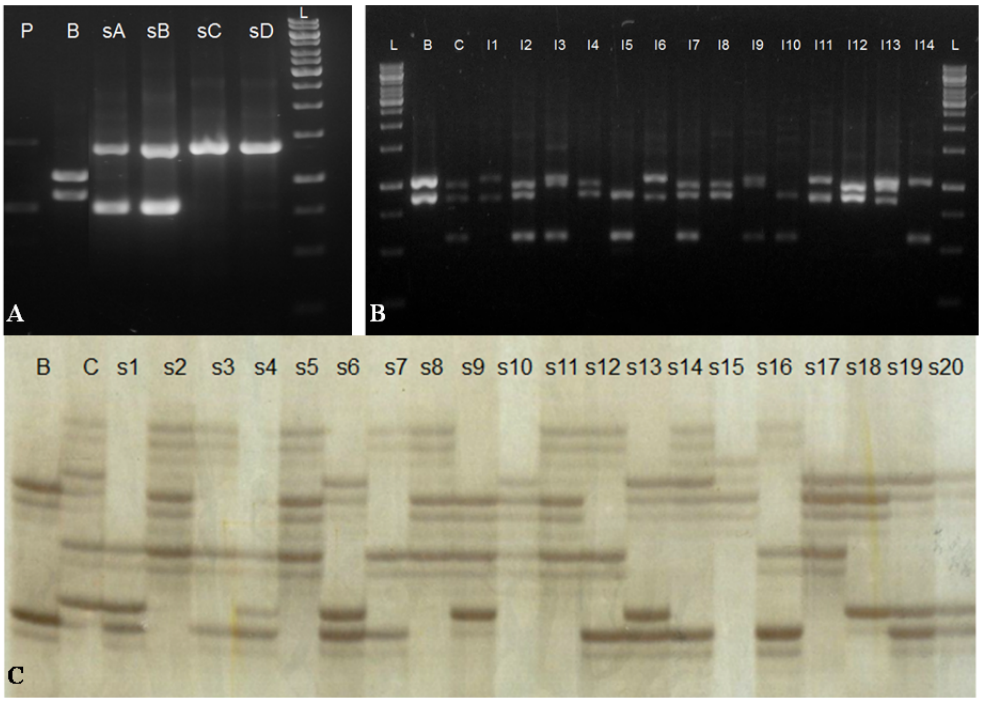

Figure 2. Results for seedling characterization from two interspecific crosses. (**A**) PCR analysis of DNA from 4 seedlings of the cross 'Pontaleb' (P) × 'Bing' (B), amplified with consensus primers for *S*-RNase gene. (**B**) PCR analysis of DNA from 14 seedlings from the cross of 'Bing' (B) x 'Cab 6P' (C), also amplified with consensus primers for *S-RNase* gene. (**C**) Microsatellite profile of 20 segregants obtained from the cross of 'Bing' × 'Cab 6P' with the microsatellite marker PMS-67. L: GeneRuler ™1kb DNA Ladder (Thermo Fischer Scientific, West Palm Beach, FL).

CONCLUSIONS

These results exemplify the utility of molecular markers as tools to complement the traditional breeding techniques to make the programs more cost-effective and to allow for a better planning of interspecific crosses.

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