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# Seed and Seedling Nursery Characteristics for 10 USDA Citrus Rootstocks

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***Abstract*. Six new hybrid rootstocks, 'US-1279', 'US-1281', 'US-1282', 'US-1283', 'US-1284', and 'US-1516', were released from the U.S. Department of Agriculture (USDA) citrus breeding program to provide improved tree tolerance to huanglongbing (HLB), the most destructive disease facing the citrus industry in the United States and many other parts of the world. Five of these new rootstocks were released based on field performance in trials with the rootstocks propagated by stem cuttings, rather than the traditional propagation using nucellar seedlings. In this study, we evaluated the fruit, seed, and seedling characteristics of these new rootstocks, along with four other USDA rootstocks of commercial importance. The study included a determination of the percentage of true-to-type and off-type seedlings by both plant morphology and simple sequence repeat (SSR) markers. All 10 rootstocks produced an acceptable number of seeds and good seedling emergence from those seeds. The rootstocks 'Swingle', 'US-802', 'US-812', 'US-1283', 'US-1284', and 'US-1516' had a high percentage of true-to-type seedlings and correspondingly good potential to be propagated by seeds. However, no true-to-type plants were observed among seedlings from the rootstocks 'US-1279', 'US-1281', and 'US-1282', indicating that economical seed propagation will be impossible for these cultivars. The 10 SSR marker sets used in this study were observed to easily differentiate the 10 rootstocks studied, and readily distinguished true-to-type and off-type seedlings among progeny from all 10 rootstock clones. This study presents information of significant value for commercial nurseries involved in propagation of citrus rootstocks, and those involved in citrus rootstock breeding and development around the world. We propose the use of these 10 SSR marker sets as readily applicable for accurate identification of most citrus rootstock cultivars and their true-to-type seedlings.***

The rootstock is an important component of a healthy and productive citrus tree, influencing the fruit yield, fruit quality, tree size, and tolerance of diseases (Bowman and Joubert, 2020; Castle et al., 2011; McCollum and Bowman, 2017; Wutscher and Bowman, 1999). HLB disease (also known as citrus greening) is arguably the most important and most destructive disease in much of the world's citrus industry. Some hybrids of trifoliate orange (*Poncirus trifoliata*) with *Citrus* spp. have been identified as tolerant to HLB when used as a scion (Albrecht and Bowman, 2019; Folimonova et al., 2009) or as a rootstock (Albrecht and Bowman, 2011, 2012; Bowman et al., 2016a, 2016b). Hybrids of this parentage are known to possess many other outstanding rootstock characteristics. As a consequence, use of HLB-tolerant rootstocks is considered one of the most effective tools currently available to combat the disease. After new rootstocks are tested and released, one of the first challenges is to obtain enough plants of the new rootstock clones in nursery propagation.

Although propagation of citrus rootstocks can be accomplished effectively by stem cuttings or micropropagation (Albrecht et al., 2017; Bowman and Albrecht, 2017), commercial propagation of citrus rootstocks usually depends on the production of genetically uniform clonal plants from seed. Within the genus *Citrus*, many species show the phenomenon of nucellar polyembryony, which means that seeds contain multiple embryos produced by ordinary mitotic division of cells of the nucellus (nucellar embryos) and no male gamete contributes to their formation (Garcia et al., 1999). For these species, some or a large portion of the seedlings will therefore be genetically identical to the seed parent. Historically, clones have been used as citrus rootstocks only when they provide a relatively high proportion of genetically uniform nucellar seedlings (Bowman and Joubert, 2020). Eliminating the zygotic plants among primarily nucellar rootstock seedling populations in the citrus nursery is important to maintain genetic homogeneity, thereby assuring growers of uniform rootstock performance in the field (Ruiz et al., 2000). In the citrus nursery, zygotic seedlings of rootstocks are eliminated, or rogued, based on visual appearance. However, separating the seedlings only by leaf morphology, size, and growth habit is not always reliable, because some seedlings of zygotic origin for particular rootstocks are difficult to visually identify (Anderson et al., 1991). If these zygotic seedlings are mistakenly used for propagation, the result can be unpredictable or reduced tree performance, including a high level of variability in tree size and health.

Several methods can be used to identify true-to-type and off-type seedlings, including isozyme analysis, random amplified polymorphic DNA (RAPD) analysis, amplified fragment length polymorphisms (AFLP) analysis, or SSR analysis (Rao et al., 2008). Isozyme analysis has limitations because of the small number of available loci in the genome and the scarce variability at those loci. RAPD and AFLP markers have limitations because of their dominant nature (heterozygous and homozygous individuals are not easily distinguished), which reduces in half the ability to detect zygotic plants in some progenies (Ruiz et al., 2000). SSR markers typically have a high number of polymorphic loci with numerous alleles (Karhu et al., 1996; Raybould et al., 1998; White and Powell, 1997), and have proven a useful tool to identify zygotic and nucellar seedlings (Russell et al., 1997).

Six new hybrid rootstocks 'US-1279', 'US-1281', 'US-1282', 'US-1283', 'US-1284', and 'US-1516' were released from the USDA citrus breeding program during 2014 and 2015 to provide improved rootstock tolerance to HLB (Bowman and McCollum, 2015; Bowman et al., 2016b). Sweet orange scions on these rootstocks demonstrated tree health and fruit productivity that was superior to the most widely used rootstocks under conditions severely challenged by HLB. All the new hybrids originated from crosses of mandarin (*Citrus reticulata*) or pummelo (*Citrus maxima*), and trifoliate orange (*Poncirus trifoliata*). At the time of release, fruits

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and seeds were not available for five of these hybrids, and little was known about their potential for seed propagation. During the 2018 season, fruiting trees were available from these new rootstocks to evaluate fruit, seed, and seedling characteristics, and determine the percentage of true-to-type and off-type seedlings by visual and SSR markers of these new rootstocks, along with four other USDA rootstocks of commercial importance. The information provided in this study is of substantial value to commercial nurseries and others interested in propagation of these rootstocks. In addition, it provides a readily applicable methodology for evaluating nursery characteristics that can be applied to other new rootstocks in the future.

## Materials and Methods

*Plant material.* Grafted seed trees of six citrus hybrid rootstocks released by the USDA in 2014–15, and four previously released rootstocks of commercial value, were used as a source of fruit and seed. Hybrids and their parentages are listed in Table 1. In January 2019, open-pollinated mature fruits from 'US-802', 'US-812', 'US-852', 'US-1279', 'US-1281', 'US-1282', 'US-1283', 'US-1284', and 'US-1516' were collected from 4-year-old grafted seed trees at the USDA, A.H. Whitmore Foundation Farm (Leesburg, FL) in a mixed planting of many genotypes, where outcrossing is likely. Fruit from the rootstock 'Swingle' were unavailable at that time. Seeds of 'Swingle' used in subsequent parts of this study were purchased from a commercial source (Lyn Citrus Seed, Arvin, CA).

*Fruit and seed characteristics.* For most of the rootstocks, four groups of eight fruits per rootstock were used to determine fruit length (mm), fruit diameter (mm), and the length/diameter ratio. The number of fruits per Florida standard 70-L field box was calculated by using an estimation of ratio of sphere volume to packing volume, which has been reported in the literature as ≈0.634 (Jaeger and Nagel, 1992; Torquato et al., 2000).

$$\text{number of fruits per field box} = \frac{(\text{volume of field box}) \times (0.634)}{(\text{fruit volume})}$$

Seeds were extracted from each fruit, and the number of seeds per fruit, seed weight (mg), and number of seeds per 50 mL were determined, and the number of seeds per liter and number of seeds per field box were calculated.

*Seedling growth.* After extraction from the fruit, seeds from each rootstock were washed, treated with a solution of 8-quinolinol sulfate (10 $g \cdot L^{-1}$; Sigma Chemical Co., St. Louis, MO), air dried, and stored at 4 °C until they were sown. For most rootstocks, six groups of 49 seeds were planted in the last week of Mar. 2019. Before planting, seedcoats were removed individually by peeling, and seeds disinfected for 20 min with 1% (v/v) sodium hypochlorite (Clorox Co., Oakland, CA) and containing 0.01% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO), and then rinsed with water. For some rootstocks, fewer than 294 seeds were available, and replications were adjusted accordingly (see Table 1). Seeds were planted into racks of 3.8 × 21-cm cone cells (Cone-tainers; Stuewe and Sons, Tangent, OR) containing steam-sterilized soilless potting medium (Pro Mix BX; Premier Horticulture, Inc., Quakertown, PA), with one seed per cell. Seedlings in cone cells were maintained in a temperature-controlled greenhouse at the U.S. Horticultural Research Laboratory (Ft. Pierce, FL) and irrigated as needed, alternating between water and water-soluble fertilizer mix of 20N–10P–20K (Peters Professional; The Scotts Company, Marysville, OH), applied with a proportioner at a rate of 400 $mg \cdot L^{-1}$ N.

*Seedling assessment.* After 80 d, seedling emergence above the soil and number of multiple seedlings were determined, and seedlings were identified as true-to-type (morphologically identical to the clonal source plants) or off-type by visual assessment from two individuals with combined experience of 30 years in citrus nursery work. Visual assessment was based on leaf morphological traits and plant growth rate. Only seedlings that were at least 30% the height of typical seedlings for that cultivar were considered as possible true-to-type seedlings, to avoid the uncertainty with visually assessing very small and stunted plants.

*Plant tissue collection.* Eighty days after seeding, six leaves were collected from each of 24 seedlings of each rootstock, to confirm the accuracy of the visual method, using SSR markers. For most rootstocks, 14 true-to-type and 10 off-type seedlings, identified by visual assessment, were evaluated. For the rootstocks 'US-1279', 'US-1281', and 'US-1282', no obvious true-to-type seedlings were observed, so all seedlings examined with SSR analysis were visually off-type, but represented the closest to true-to-type characteristics found in each replicate set of plants. For the rootstocks 'US-802' and 'Swingle', only seven and nine visually off-type seedlings were found and evaluated by SSR, respectively. Thus, for 'US-802' and 'Swingle', 17 and 15 true-to-type seedlings were evaluated by SSR, respectively.

*DNA extraction.* Leaves were ground in liquid nitrogen with a mortar and pestle, and stored at –80 °C. A total of 100 mg of ground tissue was used for DNA extraction using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Table 1. Citrus rootstock cultivar names, parentage, release date, and number of seeds and replications used in the seedling study.

| Rootstock | Parentage | USDA release date | Number of seeds (reps)[z] |
|---|---|---|---|
| Swingle | *Citrus paradisi* × *Poncirus trifoliata* | 1974 | 294 (6) |
| US-802 | *Citrus maxima* 'Siamese' × *P. trifoliata* 'Gotha Road' | 2007 | 294 (6) |
| US-812 | *Citrus reticulata* 'Sunki' × *P. trifoliata* 'Benecke' | 2001 | 293 (6) |
| US-852 | *C. reticulata* 'Changsha' × *P. trifoliata* 'English Large' | 1999 | 57 (3) |
| US-1279 | *C. reticulata* 'Changsha' × *P. trifoliata* 'Gotha Road' | 2014 | 343 (7) |
| US-1281 | *C. reticulata* 'Cleopatra' × *P. trifoliata* 'Gotha Road' | 2014 | 9 (1) |
| US-1282 | *C. reticulata* 'Cleopatra' × *P. trifoliata* 'Gotha Road' | 2014 | 107 (5) |
| US-1283 | *C. reticulata* 'Ninkat' × *P. trifoliata* 'Gotha Road' | 2014 | 294 (6) |
| US-1284 | *C. reticulata* 'Ninkat' × *P. trifoliata* 'Gotha Road' | 2014 | 294 (6) |
| US-1516 | *C. maxima* 'African' × *P. trifoliata* 'Flying Dragon' | 2015 | 294 (6) |

[z]Total number of seeds planted and () number of replications.

Table 2. Primer sequences and annealing temperatures of SSR markers used in this study.

| SSR marker | Forward sequence (5′-3′) | Reverse sequence (5′-3′) | Annealing temp (°C) |
|---|---|---|---|
| M165 | CATCAAGGCATTGGTCTAGCTC | TTGGGTGGCAGAATTAGCTG | 63 |
| M172 | TGTAAGGCCGTTACCCCTCCA | TACCATCTCCCCATGTAACGCT | 63 |
| M13 | CCCTTGTTTTACGCCACTAG | CTGATCCAGATCCAACTTACG | 63 |
| M156 | CCAAGAGAATATCCGGTGGAC | AAAGTACCCTTCATGATCACCC | 63 |
| M21 | TTCTTCAGGGTGTAATCCAG | AGCAAGAGTTCTAGTGTTAGC | 60 |
| M50 | GCGGTCGCTTAGTGAACTGT | TTGAATCCCGACCTTCTACC | 60 |
| M112 | GCAAACCACACAGTTATATCCG | CTTCGATACCGACATCAGCA | 60 |
| M126 | TACGGACATCTTCTAAACCGACC | GTCTGGACTCATTTGACTTGCAC | 60 |
| M157 | GGGTTCTTTCATCTGCCGAATG | CGAGGAATCCCCAAAGCTGAAG | 61 |
| M163 | TCACGACTCTATCCCATGTC | ACAATCCGCACTACTAATCC | 61 |

*SSR marker analysis.* Based on preliminary studies, 10 SSR markers were selected for this study. The nucleotide sequences of the primers used to detect these markers are listed in Table 2. Marker analysis was performed using the Type-It Microsatellite polymerase chain reaction (PCR) kit (Qiagen) according to the manufacturer's instructions. Each reaction contained 5 ng DNA template and 2 µM each of reverse and forward primers in a total reaction volume of 25 µL. Forward primers were labeled with 6-FAM (fluorescein) or HEX (hexachloro-fluorescein) and purchased from Life Technologies Corporation (Carlsbad, CA). PCRs were performed using a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA). A first cycle of denaturation at 95 °C for 5 min was followed by 28 cycles at 72 °C for 30 s (denaturation), 60 to 63 °C for 90 s (annealing), and 72 °C for 30 s (extension), followed by a final extension step at 60 °C for 30 min. Annealing temperatures varied by primer set and are listed in Table 2.

One microliter of PCR product was mixed with 14 µL Hi-Dye formamide solution (Amresco, Solon, OH) premixed with the GeneScan Rox 500 Size Standard (Applied Biosystems, Inc., Foster City, CA). This mixture was denatured at 95 °C for 3 min, then immediately cooled to 4 °C and subjected to automated fragment analysis by an ABI 3730xl DNA sequencer (Applied Biosystems, Inc.) following manufacturer's instructions. Analyses were performed using GeneMapper 5.0 software (Thermo Fisher Scientific, Waltham, MA). Alleles were manually assigned to clear and consistent fluorescence peaks. Inconsistent fluorescence peaks, such as stutters, pull-ups, or dinosaur tails were excluded (Pan, 2006).

*SSR marker comparison of hybrids and parental species.* All 10 primer pairs were examined with known clonal source plants of the 10 rootstock cultivars and eight representatives of the parental species, 'Cleopatra' mandarin (*Citrus reticulata*), 'Sunki' mandarin (*C. reticulata*), 'Ninkat' mandarin (*C. reticulata*), 'Pandan Wangi' pummelo (*Citrus maxima*), 'Sha Tian You' pummelo (*C. maxima*), 'US-145' pummelo (*C. maxima*), 'Rich 16-6' trifoliate orange (*Poncirus trifoliata*), and 'Flying Dragon' trifoliate orange (*P. trifoliata*). For some of the rootstocks in the study, the precise parental clones are either uncertain or were unavailable for the SSR analysis.

The Polymorphism Information Content (PIC) was calculated for each marker by applying the formula

$$\mathrm{PIC} = 1 - \sum_{j=1}^{n} P_{ij}^2,$$

where *Pij* is the frequency of the *j*th allele for marker I, and summation extends over *n* alleles (Qi et al., 2012). The fragment sizes of each seedling were compared with the fragment sizes of the clonal source plant for each individual, to identify the off-type and true-to-type seedlings. Seedlings were confirmed true-to-type when all fragments were identical to those of the clonal source plant for that cultivar.

*Statistical analysis.* Data were analyzed using Statistica v.13.3 (TIBCO Software Inc., Palo Alto, CA). Comparison of the means was performed by Tukey's honestly significant difference test when *P* was smaller than 0.01.

## Results

*Fruit and seed characteristics.* There were significant differences for all fruit and seed characteristics among the rootstocks studied (Table 3). The rootstocks 'US-802' and 'US-1516' had the largest fruit and the highest number of seeds per fruit, with 44 and 36 seeds, respectively. US-1283 produced the smallest fruit (1154 fruit per field box), and there were an average of 21 seeds per fruit, resulting in the largest number of seeds (23,858) per field box of fruit for any of the rootstocks. The average number of seeds per fruit for a cultivar was generally associated with the average fruit size, with larger fruit having more seeds. The number of stored seeds per liter ranged from 2250 for 'US-1281' to 6477 for 'US-1284'.

*Visual assessment of seedlings.* Most of the rootstocks had a large percentage of emerged seedlings, with more than 95% emergence observed for the rootstocks 'US-802', 'US-812', 'US-1516', 'US-1283', 'US-1284', and 'Swingle' (Table 4). The rootstock with the lowest percentage of emerged seedlings (68.24%) was 'US-1282'.

None of the seeds from rootstocks 'US-1281' and 'US-1282' produced multiple seedlings and 100% of seedlings were identified as off-type by visual assessment. 'US-1279' seeds also produced 100% of off-type seedlings, with only 0.3% multiple seedlings per seed. The percentage of strong true-to-type seedlings ranged from 96% for 'US-802', to 0% for 'US-1279', 'US-1281', and 'US-1282'. The rootstocks 'Swingle', 'US-802', 'US-812', and the new hybrids 'US-1283' and 'US-1284' had the highest percentage of emerged seedlings (96% to 99%) and the highest percentage of strong true-to-type seedlings based on visual assessment.

Although there was a general association of the percentage of multiple seedlings and

Table 3. Fruit and seed characteristics of different citrus hybrid rootstocks.

| Rootstock | Fruit length (mm) | Fruit diam (mm) | Length/diam ratio | Number of fruit per field box | Number of seeds per fruit | Seed wt (mg) | Seeds per liter | Seeds per field box |
|---|---|---|---|---|---|---|---|---|
| US-802 | 78 a | 87 a | 0.90 c | 164 d | 44 a | 170 a | 3,070 de | 7,171 c |
| US-812 | 48 c | 53 cd | 0.90 c | 677 bc | 12 b | 158 ab | 3,308 de | 8,212 c |
| US-852 | 53 b | 58 c | 0.91 c | 502 c | 17 b | 137 ac | 2,784 e | 8,307 c |
| US-1279 | 46 cd | 47 ef | 0.96 b | 898 b | 15 b | 100 d | 5,371 b | 13,309 b |
| US-1281 | 44 cd | 48 df | 0.93 bc | 872 ab | 9 b | 112 bd | 2,250 e | 7,848 c |
| US-1282 | 46 c | 51 de | 0.90 c | 793 b | 11 b | 107 cd | 4,034 c | 8,276 c |
| US-1283 | 41 d | 44 f | 0.93 bc | 1,154 a | 21 b | 105 cd | 5,501 b | 23,858 a |
| US-1284 | 43 cd | 53 cd | 0.81 d | 786 b | 19 b | 84 d | 6,477 a | 14,971 b |
| US-1516 | 75 a | 74 b | 1.01 a | 226 d | 36 a | 138 ac | 3,526 cd | 8,187 c |

Mean separations for significant analysis of variance within columns were by Tukey's honestly significant difference test at $P \leq 0.01$.

Table 4. Seedling characteristics of different citrus hybrid rootstocks.

| Rootstocks | Emerged seedling (%) | Multiple seedling (%) | True-to-type seedling (%) | Off-type seedling (%) |
|---|---|---|---|---|
| Swingle | 96 a | 24 cd | 94 a | 4 c |
| US-802 | 99 a | 45 ab | 96 a | 2 c |
| US-812 | 98 a | 58 a | 92 a | 4 c |
| US-852 | 89 ab | 12 de | 60 b | 40 b |
| US-1279 | 75 bc | 0.3 de | 0 c | 100 a |
| US-1281 | 89 a-c | 0 de | 0 c | 100 a |
| US-1282 | 68 c | 0 e | 0 c | 100 a |
| US-1283 | 98 a | 57 a | 91 a | 4 c |
| US-1284 | 97 a | 37 bc | 86 a | 5 c |
| US-1516 | 96 a | 12 de | 63 b | 27 b |

Mean separations for significant analysis of variance within columns were by Tukey's honestly significant difference test at $P \leq 0.01$.

Table 5. Amplified alleles of simple sequence repeat markers for 10 citrus hybrid rootstocks and eight parental species.

| | Markers | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Rootstocks | M165 | M172 | M13 | M156 | M21 | M50 | M112 | M126 | M157 | M163 |
| Parent clones | | | | | | | | | | |
| Cleopatra | 214 | 263, 272 | 133, 142 | 191 | 373 | 149, 155 | 251 | 177, 185 | 242 | 250 |
| Ninkat | 214 | 260, 272 | 136, 140 | 176, 185 | 373 | 149 | 251 | 185 | 243, 245 | 250 |
| Flying Dragon | 226, 234 | 249, 253 | 128 | 170, 179 | 365, 367 | 143 | 248 | 181 | 236, 242 | 232 |
| Rich 16-6 | 226, 234 | 249, 253 | 128 | 170, 179 | 365, 367 | 143, 155 | 248 | 181 | 236, 242 | 232 |
| Pandan Wangi | 206, 220 | 246, 252 | 134 | 182 | 361, 364 | 155 | 248 | 170 | 233 | 247, 250 |
| Sha Tian You | 206 | 252 | 130, 134, 142 | 179, 182 | 361 | 143 | 248 | 170 | 233 | 247 |
| US-145 | 220 | 247, 252 | 130, 142 | 182 | 361 | 143, 155 | 248 | 170 | 233 | 241, 250 |
| Sunki | 214 | 255, 263 | 133, 145 | 185 | 373 | 149 | 251 | 185 | 242 | 250 |
| Hybrids | | | | | | | | | | |
| Swingle | 214, 234 | 249, 252 | 128, 142 | 179, 182 | 361, 365 | 143, 161 | 248, 257 | 170, 185 | 233, 236 | 232, 250 |
| US-802 | 229, 234 | 247, 253 | 128, 134 | 179, 182 | 361, 365 | 143 | 248 | 170, 181 | 233, 236 | 232, 241 |
| US-812 | 214, 226 | 253, 263 | 128, 133 | 179, 185 | 367, 373 | 143, 149 | 248, 251 | 181, 185 | 242 | 232, 250 |
| US-852 | 214, 226 | 243, 253 | 128, 136 | 179, 191 | 367, 373 | 143, 149 | 248, 251 | 181, 185 | 242 | 232, 247 |
| US-1279 | 214, 226 | 249, 260 | 128, 136 | 179, 191 | 371 | 143, 149 | 248, 251 | 185 | 242 | 232, 250 |
| US-1281 | 214, 234 | 249, 272 | 128, 133 | 179, 191 | 373 | 143, 155 | 248, 251 | 185 | 242 | 232, 250 |
| US-1282 | 214, 226 | 249, 272 | 128, 133 | 179, 191 | 367, 373 | 143, 155 | 248, 251 | 185 | 242 | 232, 250 |
| US-1283 | 214, 226 | 249, 272 | 128, 136 | 176, 179 | 367, 373 | 143, 149 | 248, 251 | 185 | 242, 243 | 232, 250 |
| US-1284 | 214, 226 | 253, 260 | 128, 140 | 176, 179 | 365, 373 | 143, 149 | 248, 251 | 181, 185 | 242, 243 | 232, 250 |
| US-1516 | 206, 234 | 249, 252 | 130, 142 | 179, 182 | 362, 367 | 143, 155 | 248 | 170, 181 | 233, 236 | 232, 250 |

Table 6. Fragment size, number of total alleles (NTA) and polymorphism information content (PIC) of 10 simple sequence repeat (SSR) markers.

| SSR markers | Fragment size | NTA | PIC |
|---|---|---|---|
| M165 | 206–234 | 6 | 0.75 |
| M172 | 243–272 | 10 | 0.86 |
| M13 | 128–145 | 8 | 0.82 |
| M156 | 170–191 | 6 | 0.75 |
| M21 | 361–373 | 7 | 0.81 |
| M50 | 143–161 | 4 | 0.61 |
| M112 | 248–257 | 3 | 0.50 |
| M126 | 170–185 | 4 | 0.67 |
| M157 | 233–245 | 5 | 0.73 |
| M163 | 232–250 | 4 | 0.60 |

the percentage of true-to-type seedlings, there were cases in which multiple zygotic seedlings emerged from a single seed. Because of the size heterogeneity of seedlings, some seedlings did not grow large enough during the evaluation period to be defined as either true-to-type or off-type. Consequently, in Table 4, the sum of true-to-type and off-type seedlings is lower than 100% for some cultivars.

*SSR marker comparison of rootstock hybrids and parental clones.* All 10 primer pairs successfully amplified multiple alleles per marker in the hybrid rootstocks and the parental species (Table 5). The number of alleles ranged from 3 (M112) to 10 (M172) with an average of 5.7, and fragment size ranging from 128 and 373 (Table 6). In total, 57 alleles were detected, and all of the markers were polymorphic. The PIC values varied among the SSR markers from 0.50 (M112) to 0.86 (M172).

*SSR analysis for true-to-type seedlings.* The SSR analyses confirmed the results of the visual ratings to differentiate between true-to-type and off-type seedlings (Table 7). For 7 of the 10 rootstocks, seedling types were identified visually with 100% accuracy. For the other three rootstocks ('Swingle', 'US-802', and 'US-852'), the accuracy was 92% to 96%, with all of the errors involving true-to-type plants, which were wrongly classified by visual assessment as off-type.

## Discussion

This study presents information of significant value for commercial nurseries involved in propagation of the new citrus rootstocks, growers interested in planting trees on those rootstocks, and researchers involved in citrus rootstock breeding and development. Citrus rootstocks that produce fruit with a large number of seeds and good seedling emergence are preferred by nurseries. All six of the new rootstocks, along with the commercial standard rootstocks, showed good results for these traits.

Probably of even greater importance for a rootstock cultivar to be effectively propagated by seed, a high proportion of the seedlings must be strong growing and genetically identical to the parent cultivar. Nucellar polyembryony, which produces uniform and clonal (true-to-type) seedlings from a single seed, has traditionally been a major factor for selecting citrus rootstocks in breeding programs (Bowman and Joubert, 2020). This trait continues to be valuable for a new rootstock to have commercial success, even though alternative methods of vegetative propagation allow for potential commercialization where nucellar seedlings are never produced. The proportion of true-to-type seedlings of the six new rootstocks ranged from 0% to 91%, with the rootstocks 'US-1283', 'US-1284', and 'US-1516' having a high percentage of true-to-type seedlings and good potential to be propagated by seeds. In contrast, no true-to-type seedlings were recovered from the rootstocks 'US-1279', 'US-1281', and 'US-1282', indicating that economical seed propagation will be impossible for these cultivars. Alternative propagation by stem cuttings and micropropagation (Albrecht et al., 2017; Bowman and Albrecht, 2017) will be necessary for any commercial use of these three rootstocks.

The SSR markers used in this study amplified a large number of alleles. The value of the PIC indicates the effective number of alleles that can be detected per marker in a set of individuals (Chandra et al., 2014). The average PIC value in this study was 0.71, suggesting that the SSR markers used were very effective in detecting alleles and should be effective in distinguishing between the rootstock clones (Botstein et al., 1980).

In addition, the SSR markers used in this study were demonstrated to also be effective for distinguishing between true-to-type and off-type seedlings. Although SSR marker analysis is not economically feasible for use as routine practice in a commercial nursery, it is a valuable tool for verifying the proportion of true-to-type seedlings emerging from seeds of new rootstocks, and to help define the morphological traits to be used for visual roguing in the nursery. Understanding the genetic uniformity among seedlings of each rootstock is essential to choose the best method for propagation, avoid large economic losses in the nursery, and minimize the catastrophic damage to field plantings that can result from planting trees that have been propagated on off-type and inferior zygotic seedlings.

The SSR markers described here also appear to have value to verify rootstock cultivar identity in which there may be confused or mislabeled clonal lines, seed source trees, or batches of seed. The cost of using SSR evaluation for seed trees or groups of seedlings would be substantial and prohibitively expensive for routine use. However, when mistaken identity is suspected, using SSR markers to validate rootstock identity could be used to eliminate the risk of catastrophic tree loss in the field and limit legal liability.

It has been previously noted that the dominant trifoliate leaf trait of *Poncirus* over the monofoliate trait of *Citrus* (Soost and Cameron, 1975) allows for easy visual identification of zygotic hybrid seedlings (Chen

Table 7. Percentage of true-to-type (TTT) and off-type (OT) citrus seedlings identified by visual assessment and by simple sequence repeat (SSR) marker analysis, and correspondence of the two methods.

| Rootstocks | Number of seedlings | Visual | | SSR | | Correspondence (%) |
|---|---|---|---|---|---|---|
| | | TTT | OT | TTT | OT | |
| Swingle | 24 | 15 | 9 | 17 | 7 | 92 |
| US-802 | 24 | 17 | 7 | 19 | 5 | 92 |
| US-812 | 24 | 14 | 10 | 14 | 10 | 100 |
| US-852 | 24 | 14 | 10 | 15 | 9 | 96 |
| US-1279 | 24 | 0 | 24 | 0 | 24 | 100 |
| US-1281 | 8 | 0 | 8 | 0 | 8 | 100 |
| US-1282 | 24 | 0 | 24 | 0 | 24 | 100 |
| US-1283 | 24 | 14 | 10 | 14 | 10 | 100 |
| US-1284 | 24 | 14 | 10 | 14 | 10 | 100 |
| US-1516 | 24 | 14 | 10 | 14 | 10 | 100 |

et al., 2008). In our study, with trifoliate-leaved first-generation hybrids of *Poncirus trifoliata* with *Citrus* spp., most zygotic seedlings had a distinctly different leaf shape from the true-to-type nucellar seedlings, and therefore allowed for relatively reliable visual identification of true-to-type seedlings. It should be noted that true-to-type seedlings of rootstocks that are not F1 hybrids of *Citrus* × *Poncirus* are often much more difficult to identify by morphology. In either case, SSR marker analysis can provide good validation of the proportion of rootstock seedlings that are true-to-type. SSR marker analysis also can help distinguish among rootstocks of similar parentage (and morphology) when correct identity is in question.

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