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Phylogeny analysis of 25 apple rootstocks using RAPD markers and tactical gene tagging

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Abstract RAPD (random amplified polymorphic DNA) markers were used to fingerprint eight commercially available apple rootstocks (Nertchinsk, Northern Spy, Osman, Heyer 12, M.1, M.9, M.26 and MM.106), 10 winter hardy offsprings derived from the cross of Nertchinsk × M.9, six winter hardy offsprings derived from the cross of Nertchinsk × M.26 and one winter hardy offspring derived from each of the two crosses between Osman × Heyer 12 and Northern Spy × M.1. Phylogeny analysis using parsimony allowed us to draw the genetic relationship between these lines using only RAPD markers data. The resulting cladogram was compared to the true genetic relationship between these lines in order to assess the efficiency of RAPD markers in determining accurately the phylogenetic relationship. We also developed a DNA fingerprinting system based on 13 informative RAPD loci amplified by five RAPD primers that allowed the rapid identification of apple rootstocks.

Key words RAPD · DNA fingerprinting · Phylogeny Apple rootstocks · Identification key

Introduction

Apple is the most widely grown tree fruit crop, with a production of 24 million bushels worldwide in 1991. In Quebec, apple production represented 19% of the total fruit production in 1990 (91,654 tons) with a total value of $18.9 million (Bergevin et al. 1991). The main breeding objectives for apple rootstocks are the improvement of growth habit and winter survival. Breeding generally consists of performing a large number of crosses, but selection is delayed by up to 10 years. The long generation time and the quantitative nature of most of the important traits have hindered classical genetic studies in apple. Biochemical markers such as isozymes have been extensively studied in apple and proposed as tools for cultivar identification as well as genetic markers to assist breeding programs (Vinterhalter and James 1983, 1986; Weeden and Lamb 1985; Weller and Constrante 1986; Riemenschneider et al. 1988; Bournival and Korban 1987; Manganaris and Alston 1989). However, the paucity of informative isozymes in apple often limits their usefulness. More recently, new classes of genetic markers based on the direct assessment of DNA variations has been proposed for apple genetic studies. RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) markers have all been proposed as means to construct genetic maps, fingerprint varieties and assist apple breeders in selecting desirable genotypes (Nybom 1990a, b, 1992; Nybom and Schaal 1990; Nybom et al. 1990; Watillon et al. 1991; Koller et al. 1993; Ichikawa et al. 1991).

In the study presented here, we used 101 RAPD markers to fingerprint 25 apple rootstocks which included eight commercial apple rootstocks (Nertchinsk, Northern Spy, Osman, Heyer 12, M.1, M.9, M.26 and MM.106), 10 winter hardy offsprings derived from the cross of Nertchinsk × M.9, six winter hardy offsprings derived from the cross of Nertchinsk × M.26 and one winter hardy offspring derived from each of two crosses, Osman × Heyer 12 and Northern Spy × M.1 (Granger et al. 1991). Phylogeny analysis using parsimony had allowed the precise genetic relationship between these genotypes to be drawn using only RAPD marker data. The resulting cladogram was compared to the true genetic relationship between these lines in order to assess the efficiency of RAPD markers in determining accurately phylogenetic relationship. We also constructed an identification key based on RAPD markers to distinguish and classify apple rootstocks, and to avoid

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identification errors caused by the inherent instability of RAPD reactions.

Materials and methods

Plant materials

The 25 apple rootstocks and their pedigree are described in Fig. 1. They are commercial rootstocks (*Malus pumila*) and some of their winter hardy F_1 progenies. Rootstock Nertchinsk is a *Malus baccata* line used to introgress the winter hardiness trait into commercial rootstocks. All the lines were maintained as scions grafted on MM.106 rootstock and grown in a greenhouse.

RAPD and Phylogeny analyses

Microextraction of DNA from 5-mm leaf discs and conditions for RAPD reactions were as described previously (Cheung et al. 1993). RAPD primers were 10 nucleotides long and purchased from Operon Technologies (Alameda, Calif.).

For the RAPD reactions, 25 ng of DNA were used as template in a final volume of 25 µl reaction buffer containing 10 m*M* TRIS-HCl (pH 8.2), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.02% gelatin, 200 µ*M* of each dNTP (Pharmacia), 0.2 µ*M* of primer and 1 unit of Amplitaq (Cetus). The DNA amplifications were performed in a Hybaid™ thermal reactor programmed as follow: 30 s at 94°C (ramping 5.0), 42°C for 10 min (ramping 3.0) for 1 cycle; 1 s at 50°C (ramping 5.0), 45 s at 72°C (ramping 1.0), 5 s at 94°C (ramping 1.0) and 30 s at 42°C (ramping 3.0) for 45 cycles; and finally, 7 min at 72°C for 1 cycle.

The DNA amplification products were analyzed by electrophoresis on 1.4% agarose gel containing ethidium bromide (0.5 µg/ml) in 1×TAE buffer for 3 h at 5 V/cm and recorded by UV photography.

Twenty RAPD primers were used in this study. Only clearly amplified DNA bands were scored; a total of 101 loci could be scored unambiguously. Reproducibility of the amplification profiles was tested for each primer on DNA from two independent microextractions for each line. Only bright and reproducible DNA bands were retained for analyses. DNA bands were scored as present or absent in each of the 25 lines. The name and sequence of the primers are listed in Table 1.

Phylogeny analysis using maximum parsimony was performed on the raw data using the software developed by D. Swofford ("PAUP" for the Macintosh, v 3.1.1; Swofford 1993). Each informative DNA fragment was considered as a locus. Nertchinsk, being a different species, was set as an outgroup, and a heuristic search was performed using tree bisection reconnection, branch swapping and no topological constraints options. All of the informative RAPD markers were considered as unordered characters.

Table 1 List of the RAPD primers used to characterize 25 rootstock accessions of apple, the number of loci detected by each one and their sequence

Primer	Sequence (5′-3′)	Number of loci detected
A-01	CAGGCCCTTC	8
A-02	TGCCGAGCTG	9
A-04	AATCGGGCTG	7
A-17	GACCGCTTGT	9
A-18	AGGTGACCGT	3
B-01	GTTTCGCTCC	1
B-07	GGTGACGCAG	6
B-08	GTCCACACGG	9
B-09	TGGGGGACTC	1
B-13	TTCCCCCGCT	1
B-17	AGGGAACGAG	5
B-18	CCACAGCAGT	6
B-19	ACCCCCGAAG	4
C-02	GTGAGGCGTC	7
C-04	CCGCATCTAC	1
C-05	GATGACCGCC	7
C-08	TGGACCGGTG	4
C-09	CTCACCGTCC	3
C-10	TGTCTGGGTG	3
E-06	AAGACCCCTC	7

Identification Key

A subset of the best DNA amplification patterns obtained with five RAPD primers was selected, and DNA bands representing 13 loci were scored as present or absent. The sizes of the 13 DNA fragments were determined by comparing their gel migration to that of a 1-kb DNA ladder in the same gel. These 13 loci were used to draw the DNA amplification profile of the 25 rootstocks.

Results

RAPD analyses

Twenty RAPD primers that detected a total of 101 informative loci were used to characterize the 25 apple root-

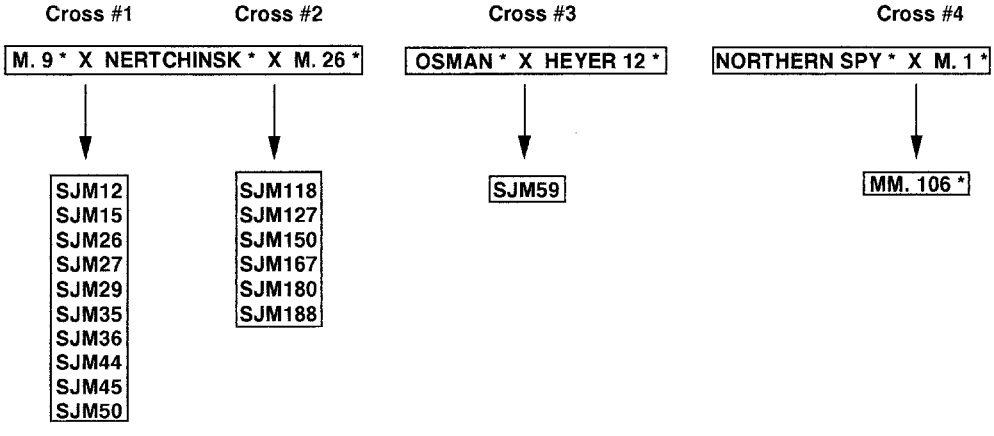

Fig. 1 Diagram of the pedigree showing the relationship of eight commercial apple rootstocks and their winter hardy F_1 progenies from four crosses (crosses 1 to 4). The eight commercial rootstocks are marked by *asterisks*

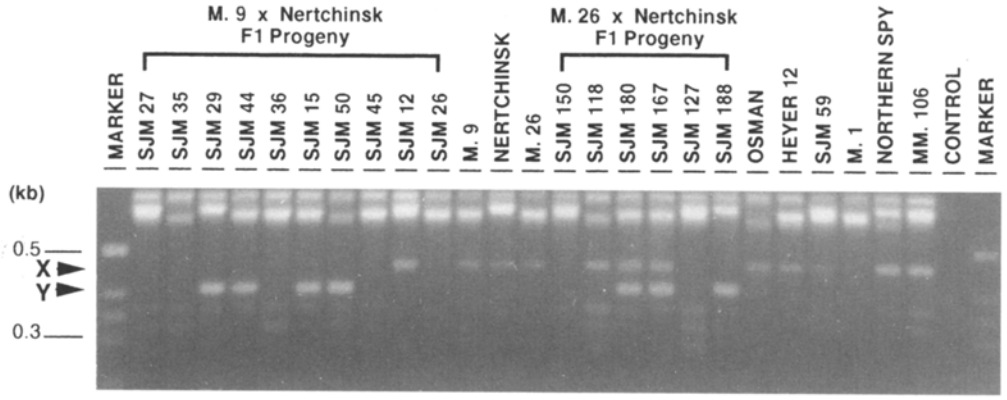

Fig. 2 Partial results obtained with RAPD primer OPA-17 on 25 apple rootstocks that demonstrate heterozygosity in parental lines and heterogeneity between the Nertchinsk plant we used for our analyses and the original Nertchinsk used for the crosses. Amplified fragment marked *X* is present in M. 9, Nertchinsk and M. 26, but is segregating in their F_1 progenies. Amplified fragment marked *Y* is absent in M. 9, Nertchinsk and M. 26, but is segregating in the F_1 progenies of both crosses M. 9 × Nertchinsk and M. 26 × Nertchinsk. The molecular weight markers are the *first* and *last* lanes, and the lane labeled *control* is the result of RAPD reaction performed without a DNA template

stocks and to separate them into distinct clades. An average of four infomative loci could be scored unambiguously for each RAPD primer, although the number of loci detected per RAPD primer varied greatly (Table 1). An example of the RAPD results is shown in Fig. 2. We also found that there was a high level of heterozygosity within the commercial rootstocks based on the DNA amplification of the F_1 plants. In some cases, both parents displayed the amplified DNA band but the F_1 progeny segregated for the band, hence one or both parents must have been heterozygous at the locus. In Fig. 2, the DNA fragment labeled "X" is present in M.9, Nertchinsk and M.26 but segregating in their F_1 progenies.

During this analysis, we also found that the plant we used as the Nertchinsk line could not have been the actual plant used to make the cross between Nertchinsk×M.9 and Nertchinsk × M.26 because some DNA bands were absent in both parents but segregating in F_1 progeny. For example, the DNA fragment labeled "Y" in Fig. 2 was absent in M.9, Nertchinsk and M.26, but had segregated in the F_1 progenies of both the M.9 × Nertchinsk and M.26 × Nertchinsk crosses. After repeating the DNA extractions and the RAPD analysis several times, the same results were obtained. However, in the other crosses where F_1 progenies were available, RAPD data were always consistant; all of the DNA bands that were observed in the F_1 progeny were present in one or the other parental line.

Cladogram of apple rootstocks

Phylogeny analysis using parsimony was performed with the data of 101 RAPD loci on the 25 apple rootstocks. A heuristic search yielded a single most parsimonious tree of length 375 after trying 31240 rearrangements. Each line was resolved as a single branch; no polytomy (branches of length zero) remained. A cladogram depicting the genetic relationship between the apple rootstocks is shown in Fig. 3.

There is a perfect agreement between the cladogram generated with the RAPD data and the real genetic relationship between the 25 apple rootstocks (see Figs. 1 and 3). We are therefore confident that this cladogram represents the true genetic relationship between the 25 apple rootstocks we analyzed. As expected, Nertchinsk, being a different species, is the most genetically distant to all the other accessions. M.9 and M.26 are the most closely related, and the F_1 progenies from the crosses Nertchinsk × M.9 and Nertchinsk × M.26 are all intermediates between Nertchinsk and M.9 and M.26 (Fig. 3). Similar results were obtained with the F_1 progenies of the crosses between Osman × Heyer 12 and Northern Spy × M.1. The growth type and cold hardiness of rootstocks are also indicated in Fig. 3 to demonstrate the potential use of this cladogram as a tool to identify the best parents to improve a specific trait in breeding programs (see discussion).

A key to identify apple rootstocks

It is becoming widely known that amplification results obtained with one RAPD primer on the same genotype can vary between laboratories, thermocyclers, source of polymerase, batches of reagents and DNA preparation (Penner et al. 1993). In order to gain a better control on this inherent instability, we constructed a key to classify apple rootstocks on the basis of a selected subsample of RAPD primers. Primers OPA17, OPA18, OPB18, OPB13 and OPC02, which provide 13 informative loci, were selected on the basis that they produced the most clearly amplified DNA bands on all accessions. A diagram of the amplification profile of each apple rootstocks at the 13 selected RAPD loci is shown in Fig. 4.

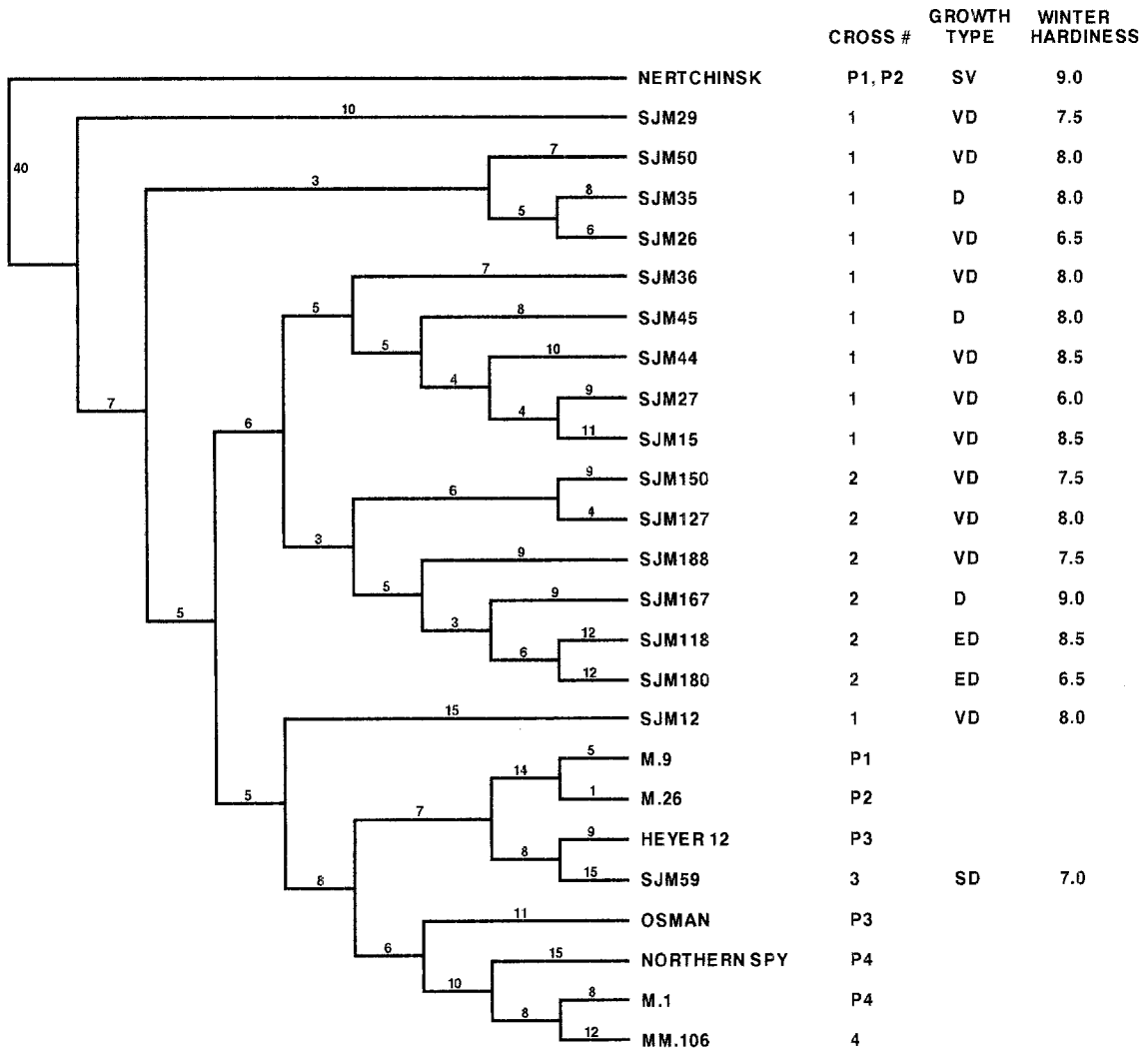

Fig. 3 Cladogram based on RAPD data showing the genetic relationship between 25 apple rootstocks. *Numbers* on the branches indicate genetic distances. Cross numbers are as in Fig. 1. Phenotypic data for growth type and winter hardiness of the offsprings are indicated at *right* and were obtained from a previous study (Granger et al. 1991). Parental lines are labeled as *P* followed by the cross number. F_1 progenies are labeled only by the cross number. For example, Nertchinsk and M. 9, parents for cross 1, are denoted by *P1*; Nertchinsk and M. 26, parents for cross 2, are denoted by *P2*. All SJM lines and MM. 106 are F_1 progenies from the cross specified by the corresponding number. Growth type describes the height of the tree obtained on the rootstock: *ED* extremely dwarfing, *VD* very dwarfing, *D* dwarfing, *SD* semi-dwarfing, *SV* semi-vigorous. Winter hardiness has been assessed for each of the F_1 offsprings with quantitative scores 1 to 9: 1 being extremely susceptible to cold conditions, and 9 being extremely winter hardy

Discussion

DNA fingerprinting of plant varieties is best achieved in vegetatively propagated species such as apple, strawberries and potato. Although these species are highly heterozygous, they are homogeneous within cultivars or lines. Our study demonstrated that RAPD markers could be used for fingerprinting apple rootstocks and lines derived from them. In addition, we unexpectedly found that the plant we used as the Nertchinsk line could not have been the actual parent used to make the cross between Nertchinsk × M.9 and Nertchinsk × M.26 because some DNA bands were absent in both parents but segregating in the F_1 progeny. Careful review of the origin of the plant that we received, however, indicated that Nertchinsk has been propagated through seeds at one time. It is therefore most likely that the Nertchinsk we used is a sister line of the original Nertchinsk.

We constructed a cladogram representing the genetic relationship between 25 apple rootstocks from the data of 101 RAPD loci. This cladogram corresponds to the real genetic relationship between the rootstocks. The cladogram can also be used to select the most appropriate parental lines to improve agronomic traits (Fig. 3). We placed in this figure two agronomic traits that were scored in a previous study on the F_1 progenies (see Granger et al. 1991) to demonstrate this application; these are growth type and winter hardiness. For example, SJM118 and SJM180 are both extremely dwarfing apple rootstocks. They also are genetically very close. If further improvement of the

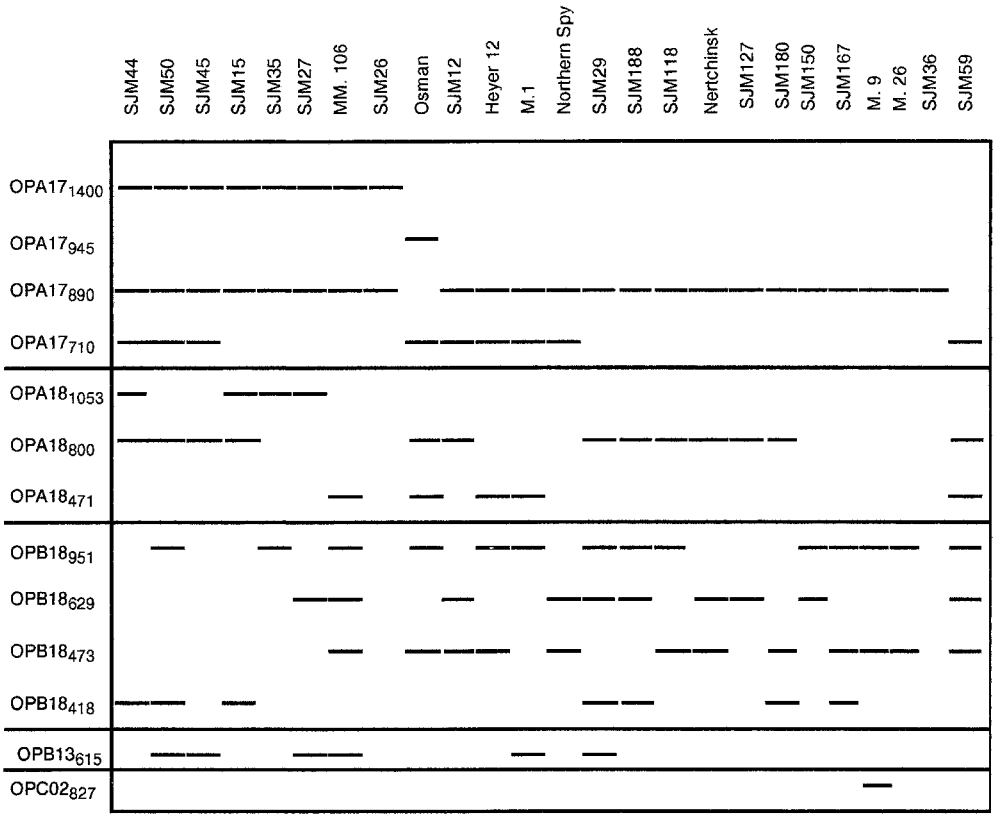

Fig. 4 Diagram of the fingerprinting profile to identify the 25 apple rootstocks using 13 selected unambiguous loci amplified with five RAPD primers. The *numbers* in *subscripts* following the name of the primers indicate the sizes in base pairs of the amplified fragments from the selected loci

dwarfing trait is desired, it is unlikely that it will be achieved using these two lines as parents. Without this cladogram, several other lines which are classified as very dwarfing could be used as one of the parental lines with either SJM118 or SJM180. With the cladogram, only SJM29, SJM50 and SJM26 are most likely to generate sufficient genetic variability to further improve this trait. We believe the same principle can be used for other complex agronomic traits, such as winter hardiness, if more markers are used.

The cladogram can be used to identify DNA markers that are potentially linked to a trait of interest when the trait is encoded by one or two genes. DNA polymorphisms that are unique to the lines which carry the desirable allele for the trait of interest are identified by sequentially eliminating DNA markers that are polymorphic between the lines that carry the desirable allele. The cladogram is useful at this point since it permits identification of those lines that carry the desirable allele but are very distant genetically; these lines are the most informative for eliminating unlinked DNA markers since some of the few alleles they have in common are likely to be linked to the trait of interest. This subset of DNA markers is then screened to select those that are polymorphic with the lines that do not carry the desirable allele. Again the cladogram is useful for identifying the lines that do not carry the same allele for the trait of interest but are very close genetically. These lines are equivalent to nearly isogenic lines. The remaining DNA markers potentially linked to the trait of interest are those which are monomorphic between the lines that carry the desirable allele and, at the same time, polymorphic with the lines that do not carry the desirable allele. We have successfully applied this approach to tag the self-incompatibility locus in canola (Cheung et al. in preparation). We call this targetted genetic mapping approach "Tactical Gene Tagging".

We selected five primers that detected 13 informative loci to make a fingerprint key based on RAPD markers. Vegetative propagation of commercial apple rootstocks greatly simplifies this identification system. Five PCR reactions are necessary to separate the 25 apple rootstocks. This key has the advantage of avoiding misscoring since such a mistake will yield more than one solution or no solution. It will also indicate where the potential error occurred. For example, if locus OPB18$_{473}$ was not scored correctly in Nertchinsk, the key would indicate two potential identifications (Nertchinsk and SJM127); if either locus OPA18$_{800}$, OPA17$_{890}$ or OPB18$_{629}$ was not scored correctly for Nertchinsk then no solution would be found, and in these cases it would indicate which locus had possibly been misscored. Although most errors in scoring can be handled in this manner, double errors will yield many more possibilities and can lead to misidentification. The system is rapid and lends itself to routine identification and certification of commercial rootstocks. As more commercial rootstocks are analyzed, the key will be easily expanded by using more RAPD primers.

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