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Development of a novel and efficient strategy for practical identification of *Pyrus* spp (Rosaceae) cultivars using RAPD fingerprints

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ABSTRACT. Accurate and reliable cultivar identification of crop species is essential to guarantee plant material identity for purposes of registration, cultivar protection and production. To facilitate identification of plant cultivars, we developed a novel strategy for efficient recording of DNA molecular fingerprints in genotyped plant individuals. These fingerprints can be used as efficient referential information for quick plant identification. We made a random amplified polymorphic DNA (RAPD) marker analysis of 68 pear cultivars. All pear genotypes could be distinguished by a combination of eight 11-mer primers. The efficiency of the method was further verified by correct identification of four cultivars randomly chosen from the initial 68. The advantages of this identification include use of fewer primers and ease of cultivar separation by the corresponding primers marked on the cultivar identification diagram. The cultivar identification diagram can efficiently serve for pear cultivar identification by readily providing the information

needed to separate cultivars. To the best of our knowledge, this is the most efficient strategy for identification of plant varieties using DNA markers; it could be employed for the development of the pear industry and for the utilization of DNA markers to identify other plant species.

Key words: Pear; RAPD; Cultivar identification; Molecular markers

INTRODUCTION

Cultivar identification and characterization are the first steps of any fruit introduction and improvement program. Breeding efforts would be facilitated by information on the genetic diversity of available germplasm resources, such as lines available from commercial seed sources. Also, an effective method for cultivar identification and fingerprinting is essential for intellectual property protection and for early identification of seedlings in the nursery industry.

Pear (*Pyrus* spp) is an important fruit crop worldwide. It belongs to the genus *Pyrus*, which comprises at least 22 species, among which *P. communis* (the European pear) and *P. pyrifolia* and *P. serotina* (Asian pear or nashi) are the most utilized for fruit production. These species are diploid (2X = 34) and self-incompatible, resulting in great genetic variability within the species. More than 5000 cultivars exist today, although only a small percentage are commercially cultivated (Bell et al., 1996). The existence of a very large number of rootstocks, cultivars and clones maintained by vegetative propagation reinforces the need for a reliable method of pear cultivar identification for researchers, nurserymen and growers. This is a very important issue for the fruit industry, particularly since the sale of fruit trees and planting of orchards involve major investments in time and money.

Traditional identification, which employs morphological and physiological traits, such as leaf size, maturity time and seed shape, is largely influenced by environmental conditions and usually requires that plants be grown to maturity. Assessment of these traits is also difficult and their evaluation can be subjective considering that most of these cultivars are related. In addition, evaluation of morphological characters is very time consuming and results are not readily transferred from one environment to another (Baird et al., 1996). In the recent past, biochemical markers, such as isozymes and seed proteins, have been widely used, although their use has often been limited by the low frequency of polymorphisms in many plant species. The number of isozyme systems required increases with the number of cultivars to be evaluated.

The advent of DNA markers has led to their application in the identification of plant cultivars, which is one of the main uses of DNA marker. Unlike biochemical markers with limited polymorphism and spatial-temporal variations, DNA-based makers are not affected by physiology or the environment and can be used to identify cultivars at any stage of development. Recently, various DNA-based markers have been developed and used for studies in genetic diversity, relationships, and origins of cultivars (D'Onofrio et al., 2009; Melgarejo et al., 2009; Cheng and Huang, 2009; Elidemir and Uzun, 2009; Papp et al., 2010). Among these markers, random amplified polymorphic DNA (RAPD) (Williams et al., 1990) markers are useful for cultivar analysis, with advantages of simplicity, efficiency, relative ease of execution, and non-requirement of any previous sequence information. RAPD markers have been used in cultivar identification and genetic relationship analysis of a number of fruit species, such as apple (Stark-Urnau, 2002a), litchi (Ding et al., 2000), grape (Qu et al., 1996; Corazza-

Nunes et al., 2002), cherry (Demirsoy et al., 2008), Indian cashew (Archak et al., 2003), longan (Yonemoto et al., 2006), olive (Belaj et al., 2003), pear (Stark-Urnau, 2002b; Lee et al., 2004), and pistachio (Kafkas et al., 2006). Despite all these advantages, there is no a single-efficient strategy for applying DNA markers in cultivar identification. The main reason for this situation is a dearth of practical analysis strategies for DNA fingerprints. The popular analysis techniques for DNA banding patterns known as cluster analyses are not efficient in cultivar or species separation. This has made the practice of utilizing DNA markers in crop and seed identification unpopular.

We developed a new strategy that makes the identification of pear cultivar a practical, efficient and reliable.

MATERIAL AND METHODS

Plant materials

Young leaves of 68 selected pear cultivars were collected from the Jiangsu Academy of Agricultural Sciences, Jiangsu, China (Table 1). The RAPD primers were synthesized by Shanghai Invitrogen Biotechnology company.

Table 1. Name and origin of the pear cultivars separated using RAPD fingerprints.

No.	Cultivar	Origin	No.	Cultivar	Origin
1	Eli 2 hao	Hubei	35	Wujiuxiang	Liaoning
2	Xizilv	Zhejiang	36	Jinfeng	Liaoning
3	Cili	Shandong	37	Zaosu	Liaoning
4	Meigetsu	Japan	38	Wakahikari	Japan
5	Cuiguan	Guangxi	39	Jinli	Shanxi
6	Manao	Henan	40	Huanghuali	Zhejiang
7	Chikusui	Japan	41	Yulvxiang	Shanxi
8	Yaguangli	Hebei	42	Jinshui 2 hao	Hubei
9	Hosui	Japan	43	Chonghuadali	Hubei
10	Hanareum	Southkorea	44	Pingguoli	Liaoning
11	Kousui	Japan	45	Long 19	Sichuan
12	Jinchuanxueli	Sichuan	46	Zuijinxiang	Liaoning
13	Hanxiang	Jilin	47	Zaomeisu	Liaoning
14	Jinhua 4 hao	Sichuan	48	Longquansuli	Sichuan
15	Weiningdahuang	Guizhou	49	Dangshansuli	Anhui
16	Starkrimson	USA	50	Jinhua	Sichuan
17	Korla xiangli	Xinjiang	51	Xinyali	Hebei
18	Jinshui 3 hao	Hubei	52	Zaojinxiang	Liaoning
19	Bayuexue	Hebei	53	Jinxingli	Henan
20	Qiyuesu	Henan	54	Guiguan	Zhejiang
21	Huajin	Liaoning	55	Aixiang	Liaoning
22	Zaohuang	Neimenggu	56	Nangetsu	Japan
23	Beixin	Liaoning	57	Kisui	Japan
24	Shinsei	Japan	58	Shinseiki	Japan
25	Kikusui	Japan	59	Huawang	Shandong
26	Nanguoli	Liaoning	60	D'Anjou	USA
27	Zhongcui	Hubei	61	Jinzhui	Hebei
28	Hongxiangmi	Henan	62	Hougetsu	Japan
29	Okusankichi	Japan	63	Puli	Xinjiang
30	Huasu	Liaoning	64	Bayuehong	Shanxi
31	Yali	Hebei	65	Jinshui 1 hao	Hubei
32	Cuilu	Zhejiang	66	Zhe 21	Zhejiang
33	Xuehuali	Hebei	67	Hangqing	Zhejiang
34	Huangguan	Hebei	68	Aigan juicy	Japan

Genomic DNA extraction and amplification of RAPD markers

Total genomic DNA of each genotype was extracted from young leaves using the modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980; Bousquet et al., 1990; Fang et al., 2006). The extracted DNA was diluted with 1X TE buffer to a final concentration of 10 ng/μL and stored at -20°C, pending use.

The reaction mixture (final volume 15 μL) contained 1.5 μL 10X buffer, 1.2 μL $MgCl_2$ (25 mM), 1.8 μL dNTP (2.5 mM), 1.2 μL primer (1.0 μM), 0.08 μL rTaq polymerase DyNAzyme (5 U/μL) and 30 ng genomic DNA. Amplification reactions were performed based on the standard protocol of Williams et al. (1990), with minor modifications. The polymerase chain reaction (PCR) was carried out in an Autorisierter Thermocycler (Eppendorf, Hamburg, Germany), programmed as follows: pre-denaturation for 5 min at 94°C, then 42 cycles each consisting of a denaturation step for 30 s, an annealing step for 1 min at annealing temperature (Table 2), and an extension step for 2 min at 72°C. Amplification was terminated by a final extension of 10 min at 72°C.

Table 2. Primers chosen for further fingerprinting of 68 pear genotypes.

Primer	Nucleotide sequence (5'-3')	Annealing temperature (°C)
Y6	GTTTCGCTCCC	44.4
Y17	AGGGGTCTTGG	36.6
Y27	GTGTGCCCCAA	44.4
Y40	AGCGTCCTCCT	43.7
Y46	ACGACCGACAT	44.4
Y47	ACGACCGACAG	44.8
Y48	ACGACCGACAC	44.8
Y60	ACCCCCGACTC	43.7

RAPD analysis

Fifty-four 11-mer RAPD primers were screened. In order to increase the credibility of the fragments, we scored only those that were very reproducible. As a result, only eight primers (Table 2) that showed well-resolved and reproducible bands were further analyzed; the rest were discarded. The PCR products were detected on 1.3% (w/v) agarose gels in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) buffer at 100 V. The gels were stained with 0.5 μg/mL ethidium bromide and photographed under ultraviolet light. Polymorphic bands among the cultivars were observed from the photographs. In order to have reproducible and clear banding patterns, all amplifications were each repeated at least thrice.

Data analysis

Only the reproducible and clear-cut polymorphic RAPD profiles were manually scored from photographic gel prints of each cultivar, and specific bands were chosen to identify the cultivars. Cultivars sharing the same band patterns were clustered into the same sub-group, and then more primers were employed to distinguish the cultivars in each sub-group. As more primers were used, more and more amplified specific bands were generated, and

eventually all the cultivars could be differentiated. Based on this, the cultivar identification diagram (CID), comprised of the bands with specific sizes used to separate the cultivars and all the related primers that generated the specific bands, was constructed for full separation of all the pear cultivars.

Test of utilization and workability of the diagram in cultivar identification

Two groups of pear cultivars were randomly chosen to verify the utilization and workability of the diagram showing the separation of the 68 cultivars. Corresponding primers that could amplify the polymorphic fragments to be used for separation of the cultivars could be readily found in the diagram. If the randomly chosen cultivars could be distinguished accurately and quickly as per the anticipated results on the whole CID, we can assure the workability and efficiency of this new approach in the identification of cultivars both for this study and also for similar study in the future. The data of the cultivar separation from this diagram can also be transferred to a database for ease of reference.

RESULTS

Cultivar identification

To establish a stable and optimistic RAPD system with high reproducibility, longer primers (11 nt) were employed, and the annealing temperatures for each primer were screened based on the quality and reproducibility of banding patterns. The primers were randomly screened from a stock of 54 11-mer primers, and once an optimal primer that could produce reproducible polymorphic bands was screened, it was utilized further in the identification of pear cultivars. After the eighth primer (Table 2) was screened and utilized, all the 68 pear cultivars could be successfully identified. Among the eight primers used, primer Y47 (Figure 1) was the first to be screened and used in identification of all the 68 pear cultivars. The electrophoresis results show that primer Y47 generated uniform, clear, and reproducible band patterns with a 1400-bp size in 42 cultivars. The lane numbers correspond to the codes in Table 1. This group of cultivars was easily differentiated from the other 26 cultivars by the presence or absence of a distinct 1400-bp band, causing all the 68 cultivars to be separated into two groups (Figure 2). Following this, the second primer Y6 (Figure 2) was chosen to further differentiate members in the two resulting groups of pear cultivars. Primer Y6 could separate the two groups of cultivars identified by primer Y47 into smaller groups. The two initial groups, comprised of 42 and 26 pear cultivars, could both be separated into two subgroups each with a band size of about 1800 bp (Figure 2). The remaining six primers (Table 2) were, step by step, screened and chosen to differentiate the pear cultivars until full separation was achieved (Figure 2). Eventually, all the 68 pear cultivars were successfully differentiated from each other by the joint use of eight different primers; the flow diagram of amplification makes the identification of these 68 cultivars an efficient, reliable, and simple process, useful for the pear industry, since there was a close connection between the specific bands, primers used and the cultivars identified.

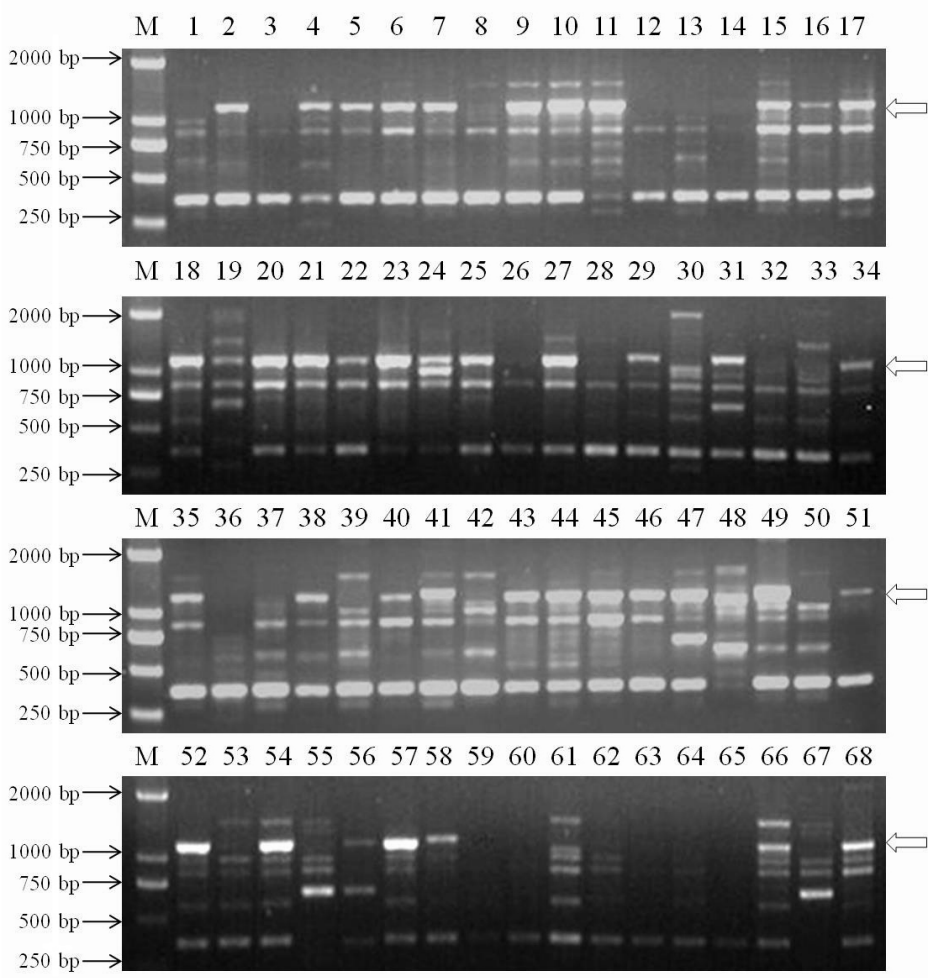

Figure 1. RAPD patterns of 68 genotypes within the genus *Pyrus* obtained with primer Y47. Horizontal arrows indicate the specific bands. The lane numbers correspond to the codes in Table 1. M = DNA size marker.

Test of use and workability of the diagram in cultivar identification

This strategy could also make DNA markers more applicable for plant variety identification. However, this study was not only aimed at generating a diagram like for cluster analysis of some cultivars; the diagram generated should be reliable and applicable for the practical identification of other pear cultivars even in future. To ascertain if this important objective had been met, verification of the utilization, workability and efficiency of the diagram in cultivar identification was done; two groups of cultivars including 'Huasu', 'Nanyue', 'Bayuehong', and 'Aiganshui' were randomly chosen to be used for the verification exercise. From the location of these cultivars in CID, it was easy to find the primer to use in separating them; the primers Y47, Y40 and Y27 were used to separate the four cultivars. PCR results show that the four pear cultivars could initially be separated into two groups by primer Y47, with a band of about 1400 bp. One group

had 'Nanyue' and 'Aiganshui', and could further be separated by primer Y27, with the 1900-bp band. The other group was made up of 'Huasu' and 'Bayuehong', and could be divided by use of primer Y40, with a band of about 1200 bp (Figure 3). Identification of the selected four cultivars using the three specific primers as anticipated indicates the usability and power of this CID. In this way, all the 68 cultivars could be successfully identified with suitable primer combinations.

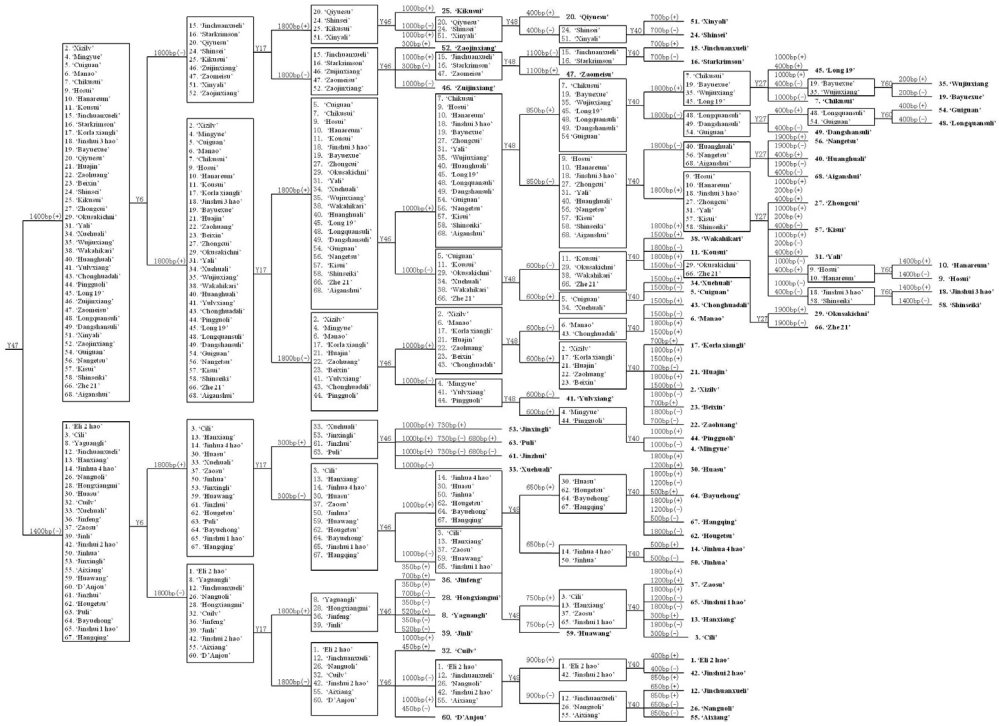

Figure 2. Cultivar identification diagram of 68 pear cultivars obtained with eight RAPD primers. Note: (+) band present; (-) band absent. The names of the cultivars in bold mean that these cultivars were completely separated.

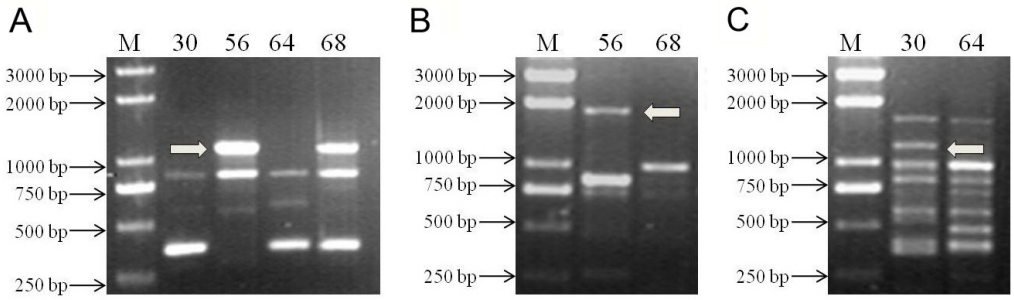

Figure 3. RAPD profiles obtained with RAPD primers. Horizontal arrows indicate the specific bands. The lane numbers correspond to the codes in Table 1. M = DNA size marker. **A.** Obtained with primer Y47. **B.** Obtained with primer Y27. **C.** Obtained with primer Y40.

DISCUSSION

One of the main purposes of plant science is to provide service to agriculture, with the application of new biological techniques to agricultural practices being very important. Even though several generations of DNA markers have been developed and used for cultivar identification (Saker et al., 2006; Chiu et al., 2010), genetic analysis (Boronnikova et al., 2007; Silvestrini et al., 2008; Bhau et al., 2009; Baysal et al., 2010), and in the process of thousands of papers published, they are not readily and efficiently used in genotyping. Whenever the need to distinguish some cultivars arises, we still do not know which primer or marker to use and how many primers or reactions are needed to identify some cultivars. This is partly due to the fact that the fingerprints have not been categorized or digitized for easy reference. In fact, no efficient approach has been developed and successfully used for cultivar identification, except where phylogenetic trees and some fingerprints were employed. The former cannot tell us which information can be used or referred to for identification of a specific cultivar, while the later cannot present all the fingerprints together and is therefore not suitable for identification of many cultivars.

Developing a strategy that can make the most use of the apparent advantages of DNA markers for easy identification of pear cultivars is very vital for the pear nursery and production industry. Towards this end, we successfully identified 68 cultivars of pear using polymorphic RAPD markers by developing and deploying a new strategy. This identification could generate a CID that clearly presents the information needed for identification of groups of pear cultivars. The CID can work just like the Periodic Table of Elements is used in chemistry and is quite advantageous due to its reliability, workability, ease of use, and flexibility, since more new cultivars can be added in as they are identified. This resource would not only provide valuable information and theoretical scientific basis on cultivar identification, genetic diversity, cultivar introduction, and genetic improvement at the molecular level, but also can be an essential requirement for granting of protection to all the new varieties through DUS (distinctness, uniformity and stability) testing (Lu et al., 2009). This new approach is both efficient and practical, with less cost, rapidness and objectivity, among other attributes. This strategy can open up the immense power of DNA markers in plant cultivar identification, and the polymorphism of each primer can be used to gradually distinguish and chart every species. Although this method does not accurately reflect the genetic relationship between the cultivars/species being identified, theoretically, the earlier the separation, the greater the genetic distance between the cultivars/species, while the genetic distance between cultivars/species separated later in a CID is much closer. This method creates a readable and recordable flow chart, making fruit crop cultivar identification much easier than before.

China is an important agricultural country and has abundant plant resources, which makes the task of distinguishing plant cultivars or varieties very important. For a long time, pollen morphology, cytology and iso-enzymes have been the main techniques used for identifying pear species and cultivars (Banno et al., 2000). However, the use of DNA marker techniques is gradually gaining ground and has elicited much interest in the fruit industry (Kim et al., 2000; Teng et al., 2001; Schiliro et al., 2001; Xuan, 2008). Our new approach is not just a simple model; it also makes DNA markers more applicable for plant variety identification in practice. It has been found to be efficient in distinguishing plant cultivars and gives reliable information for future rapid identification work. Our study drastically simplifies the identifica-

tion of plant cultivars, making it direct and as easy as measuring the length of an object using a ruler. Although a single-RAPD primer cannot distinguish all pear cultivars at the same time, this method represents a substantial increase in efficiency over previous studies. Additionally, it also reveals new evidence on the rapid identification of plant cultivars and the results further show that any two plant cultivars can be identified by the use of one specific primer. At present, we have initiated similar study to present as much identification information as possible for most fruit crop cultivars in China for the purposes of cultivar-right-protection and to provide a better service for the nursery industry.

We used RAPDs to detect many specific bands for the cultivars of the genus *Pyrus*, thus providing useful information for identification within this genus. It is, however, necessary to incorporate the critical limitations of this method when interpreting results. Principally, we have to consider the lack of complete reproducibility of the technique. In order to guarantee reproducibility, some adjustments in the annealing temperature, number of amplification cycles and concentrations of genomic DNA, magnesium and polymerase were made to the original program to improve the results. Therefore, it is very important to ascertain the conditions of the analysis, particularly the amount of DNA used.

This is a first report on using RAPD primers in sequence to identify pear cultivars. In order to verify the reliability of this theory, the verification of the workability and efficiency of the diagram in cultivar identification was vital and it gave satisfactory results. Therefore, this experiment suggests the possibility of utilizing DNA markers even in other plant species having a highly heterozygous genome, without the need for a genetic linkage map or any DNA sequence information to distinguish the cultivars. It appears to be an effective technique for conveniently developing selection markers in fruit cultivars, and it can also make DNA markers more applicable for plant variety identification in practice.

In conclusion, this new strategy is rapid, simple, and produces reliable results, since it was possible to demonstrate that a standard set of primers can be used to distinguish a large number of *Pyrus* species.

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