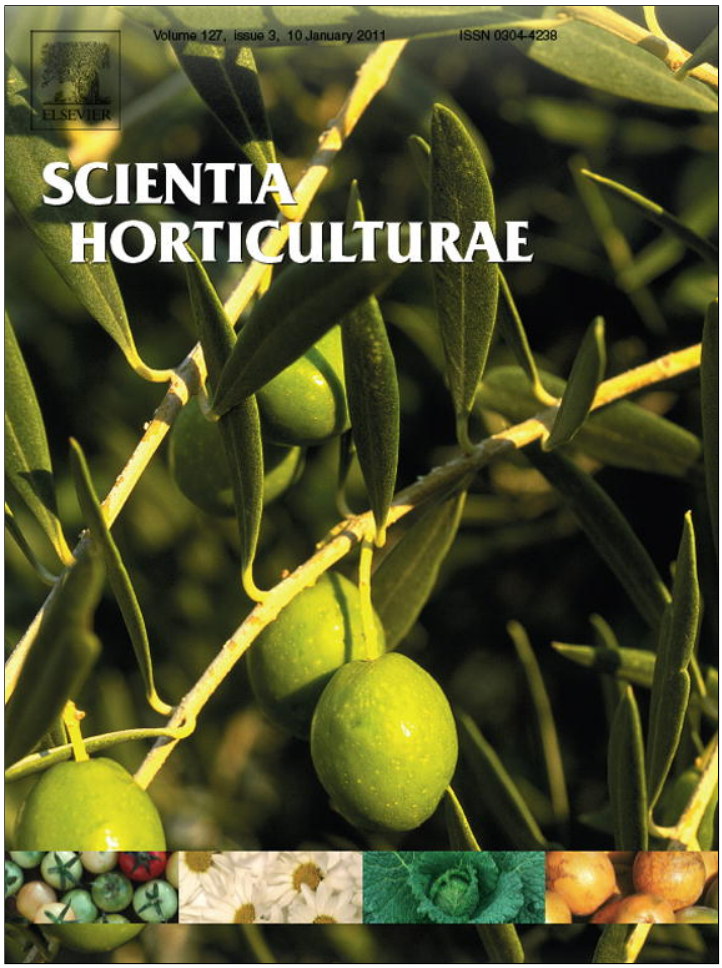

Scientia Horticulturae 127 (2011) 405–410

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Development of ITS sequence based SCAR markers for discrimination of *Paphiopedilum armeniacum*, *Paphiopedilum micranthum*, *Paphiopedilum delenatii* and their hybrids

Yung Wei Sun [a], Yu Ju Liao [a], Yin Sui Hung [a], Jia Ci Chang [a], Jih Min Sung [b,*]

[a] *Taiwan Seed Improvement and Propagation Station, Taichung County 42642, Taiwan, ROC*
[b] *Department of Food Science and Technology, Hung Kuang University, Taichung County 43302, Taiwan, ROC*

ARTICLE INFO

Article history:
Received 13 August 2010
Received in revised form
28 September 2010
Accepted 13 October 2010

Keywords:
ITS
Paphiopedilum armeniacum
Paphiopedilum micranthum
Paphiopedilum delenatii
SCAR marker

ABSTRACT

Paphiopedilum armeniacum, *Paphiopedilum micranthum* and *Paphiopedilum delenatii* are endangered orchid species. These three Paphiopedilum species and their hybrids are difficult to distinguish morphologically. In this study, rDNA-ITS (internal transcribed spacer) sequences were used to design species-specific SCAR (sequence characterized amplified regions) markers to distinguish *P. armeniacum*, *P. micranthum*, *P. delenatii* and their respective hybrids. The developed markers efficiently amplified 600 bp DNA product for *P. armeniacum* and its hybrids (SCAR-600armF/Pap-ITS2R), 300 bp product for *P. delenatii* and its hybrids (SCAR-300delF/Pap-ITS2R) and 700 bp product for *P. micranthum* and its hybrids (SCAR-700micF/Pap-ITS2R). The effectiveness of designed species-specific markers was also confirmed by using multiplex polymerase chain reaction amplification with a combination of developed three SCAR markers.

1. Introduction

Paphiopedilum armeniacum, *Paphiopedilum micranthum* and *Paphiopedilum delenatii* are orchid species native to southwestern China and Vietnam. They are endangered species and are currently listed as Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I species (Liu et al., 2006). Thus, the international trade for these naturally occurring species is forbidden. However, the artificially propagated materials and their inter-species hybrids forced by breeders can be traded for commercial purposes. As a result, many well-diverged hybrids with complicated pedigree exist in the orchid market worldwide. These artificially propagated species and their inter-species hybrids are morphologically similar occasionally, particularly in vegetative parts. In the past, the differentiation of these species and their hybrids were largely based on morphological features such as color, shape, the existence of variegated leaves and petal size (Chung et al., 2006). However, morphological diagnosis often causes conflicting ideas, because the key morphological characters lack unanimity among taxonomists. Additionally, morphological characters are frequently affected by the developmental stages of the experimental material and environmental effects (Hussain et al., 2008).

Molecular markers have proved their utility in fields like taxonomy, physiology and genetics (Soltis and Soltis, 2000; Kumar et al., 2009). Several DNA-based markers such as randomly amplified polymorphic DNA (Chung et al., 2006) and microsatellite loci (Rodrigues and Kumar, 2009) are explored to identify various orchid species. RAPD has been widely used in all kinds of genetic analysis, because of their ease of use and universal applicability. However, the major limitation of RAPD is its low reproductively (Kumar et al., 2009). Microsatellite is a high sensitive method with high reproducibility and high polymorphism. This technique suits the identification of closely related varieties (Barbara et al., 2007), but its major drawbacks are high developmental cost and the difficulty of training.

Internal transcribed spacer (ITS) region of rDNA, defined as the unit containing the ITS1 spacer, 5.8S rDNA gene and ITS2 spacer, have been shown to be a useful sequence to study phylogenetic relationships in many plant species (Soltis and Soltis, 2000; Zhang et al., 2007; Choo et al., 2009; Gulbitti-Onarici et al., 2009). This region can be readily amplified by polymerase chain reaction (PCR) with specific primers and sequenced. Because of their different rates of evolution, ITS regions have become the favored markers in evolutionary studies at different taxonomic levels (Gulbitti-Onarici et al., 2009). This technique is also proved to be a useful molecular marker for screening different species of slipper orchid (Cox et al., 1997; Zhang et al., 2007). In the present study, the plants of 32 *Paphiopedilum* species, including *P. armeniacum*, *P. micranthum*, *P. delenatii* and some of their hybrids were evaluated. The nucleotide

* Corresponding author. Tel.: +886 4 22873459; fax: +886 4 22860307.
E-mail address: sungjm@sunrise.hk.edu.tw (J.M. Sung).

0304-4238/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2010.10.009

Table 1
The primer sequences used for PCR analysis and the molecular size amplified by these sequences in *P. armeniacum*, *P. delenatii* and *P. micranthum*.

Species	Primer	Sequence of DNA marker (5′ → 3′)	Produced DNA fragment
	Pap-18SF Pap-ITS2R	TTGACTACG TCC CTG CCC TTT CTTAAACTCAGCGGGTTGCCT	890 bp
P. armeniacum	SCAR-600armF Pap-ITS2R	GCGCAGTTTTGC CCAAGA CTTAAACTCAGCGGGTTGCCT	600 bp
P. delenatii	SCAR-300delF Pap-ITS2R	CTTAACGAG GCTGTCCAG CTTAAACTCAGCGGGTTGCCT	300 bp
P. micranthum	SCAR-700micF Pap-ITS2R	GTTTACTTTGGTCACCCT CTTAAACTCAGCGGGTTGCCT	700 bp

sequences of the rDNA-ITS of *P. armeniacum*, *P. micranthum* and *P. delenatii* were compared. Several species-specific sequence characterized amplified regions (SCAR) markers for distinguishing *P. armeniacum*, *P. micranthum*, *P. delenatii* and their hybrids based on nuclear ITS sequence were also developed. Furthermore, the discrimination markers for the simultaneous detection of these three species and their hybrids by the application of multiplex-PCR methods by combining the primers designed for the SCAR markers were also examined.

2. Materials and methods

2.1. Plant material and DNA extraction

The plants of 32 *Paphiopedilum* species, including *P. armeniacum*, *P. micranthum*, *P. delenatii* and some of their hybrids were collected from different orchid nurseries located in central and south parts of Taiwan. The orchid genomic DNA was extracted from fresh leaves (0.1 g) using a plant genomic DNA purification kit (Hopegen Biotechnology Development Enterprise, Taichung County, Taiwan) according to the manufacturer's instructions. DNA concentrations were determined by measuring the absorbance of diluted DNA solution at 260 nm wavelength. The extracted DNA samples were kept at −20 °C for further use.

2.2. Development of species-specific markers

The rDNA-ITS regions (ITS1, 5.8S and ITS2) of the tested *Paphiopedilum* species were amplified using the primers Pap-18SF and Pap-ITS2R (Table 1). The primers were synthesized by Hopegen Biotechnology Development Enterprise (Taichung County, Taiwan). PCR reaction contained 200 ng of genomic DNA was carried out using Taq DNA polymerase master mix (AMP180303; Ampliqon, Copenhagen, Denmark) and the volume was made up to 25 μL with sterile double distilled water. A PCR thermocycler Px2 (Thermo Electron Corporation, Milford, MA, USA) was used for PCR reaction. The parameters for rDNA-ITS were: 94 °C for 2 min; followed by 30 cycles of 45 s at 94 °C, 45 s at 45 °C, 2 min at 72 °C, and 1 cycle of 7 min at 72 °C. PCR products were separated electrophoretically on a 1.5% agarose (Lonza Rockland, Inc., Rockland, ME, USA) gel with 50 μg ml^{-1} Safe View DNA stain (Hopegen Biotechnology Development Enterprise, Taichung County, Taiwan), with a 100 bp DNA ladder (GM100-LC, Hopegen Biotechnology Development Enterprise, Taichung County, Taiwan) providing size markers. The gel was analyzed using AlphaImager 2200 and AlphaEase FC software package (Version 4.1.0, Alpha Innotech Corporation, CA, USA). These ITS fragments were direct sequenced by Genomics BioSci. & Tech. Co. (Xizhi City, Taipei County, Taiwan).

The species-specific primers (Table 1) for three slipper orchid species were designed within ITS regions by the comparison of rDNA-ITS sequences. The amplification reactions were carried out in 25 μl containing 0.2 μM of each primer, 200 ng of template and the same buffer described above. Amplification of reactions was carried out in a thermal cycle, as described above, with the following cycling conditions: pre-denaturation at at 94° C for 2 min, followed by 30 cycles of 45 s at 94° C, 45 s at 58° C, 2 min at 72° , and final extension at 72° C for 7 min.

Fig. 1. Morphological variations in leaves and flowers of tested *Paphiopedilum armeniacum*, *Paphiopedilum micranthum*, *Paphiopedilum delenatii* and their hybrids.

Table 2
Molecular size of the PCR products produced by using using Pap-18SF and Pap-ITS2R primers and three SCAR primer pairs SCAR-600armF/Pap-ITS2R, SCAR-300delF/Pap-ITS2R, SCAR700micF/Pap-ITS2R in the tested 32 *Paphiopedilum* species.

Species	Primer name			
	Produced DNA fragment			
	Pap-18SF Pap-ITS2R	SCAR-600armF Pap-ITS2R	SCAR-300delF Pap-ITS2R	SCAR-700micF Pap-ITS2R
P. malipoense	890 bp	–	–	–
P. armeniacum	890 bp	600 bp	–	–
P. micranthum	890 bp	–	–	700 bp
P. delenatii	890 bp	–	300 bp	–
P. vietnamense	890 bp	–	–	–
P. callosum	890 bp	–	–	–
P. sukhakulii	890 bp	–	–	–
P. lawrenceanum	890 bp	–	–	–
P. urbanianum	890 bp	–	–	–
P. argus	890 bp	–	–	–
P. acmodontum	890 bp	–	–	–
P. dayanum	890 bp	–	–	–
P. sanderianum	890 bp	–	–	–
P. philippinense	890 bp	–	–	–
P. stonei	890 bp	–	–	–
P. rothschildianum	890 bp	–	–	–
P. gardineri	890 bp	–	–	–
P. supardii	890 bp	–	–	–
P. bellatulum	890 bp	–	–	–
P. villosum	890 bp	–	–	–
P. concolor, *P. bellatulum*	890 bp	–	–	–
P. concolor	890 bp	–	–	
P. barbigerum	890 bp	–	–	–
P. exul	890 bp	–	–	–
P. esquirolei	890 bp	–	–	–
P. charlesworthii	890 bp	–	–	–
P. chamberlainianum	890 bp	–	–	–
P. glaucophyllum	890 bp	–	–	–
P. primulium	890 bp	–	–	–
P. haynaldianum	890 bp	–	–	–
P. lowii	890 bp	–	–	–
P. dianthum	890 bp	–	–	–

3. Results and discussion

Many well-diverged hybrids of *P. armeniacum*, *P. micranthum* and *P. delenatii* currently exist in the slipper orchid market worldwide. These hybrids are morphologically similar in some cases (Fig. 1), particularly in vegetative parts, and are very difficult to identify accurately. The rDNA-ITS region have been used in phylogenetic analysis in plants at generic and intrageneric levels. The length and sequences of ITS regions of ribosomal RNA gene repeats are believed to be fast evolving and therefore may vary. Universal PCR primers designed from highly conserved regions flanking the ITS, relatively small size of the ITS region and high copy num-

Table 3
The PCR products using multiplex-PCR amplification of SCAR-600armF/SCAR-300delF/SCAR-700micF/Pap-ITS2R primer pairs in the tested 26 *Paphiopedilum* inter-species hybrids.

Hybrid	SCAR-600armF Pap-ITS2R	SCAR-300delF	SCAR-700micF
	Produced DNA fragment		
P. armeniacum × *P. anitum*	600 bp	–	–
P. armeniacum × *P. hangianum*	600 bp	–	–
P. armeniacum × *P. vietnamense*	600 bp	–	–
P. bellatulum × *P. armeniacum*	600 bp	–	–
P. delenatii × *P. armeniacum*	600 bp	300 bp	–
P. delenatii × *P. delenatii*	–	300 bp	–
P. delenatii × *P. hangianum*	–	300 bp	–
P. delenatii × *P. malipoense*	–	300 bp	–
P. delenatii × *P. micranthum*	–	300 bp	700 bp
P. delenatii × *P. vietnamense*	–	300 bp	–
P. emersonii × *P. armeniacum*	600 bp	–	–
P. malipoense × *P. micranthum*	–	–	700 bp
P. micranthum × *P. bellatulum*	–	–	700 bp
P. micranthum × *P. delenatii*	–	300 bp	700 bp
P. micranthum × *P. malipoense*		–	700 bp
P. micranthum × *P. rothschildianum*	–	–	700 bp
P. micranthum × *P. vietnamense*	–	–	700 bp
P. primulium × *P. armeniacum*	600 bp	–	–
P. primulium × *P. delenatii*	–	300 bp	–
P. rothschildianum × *P. delenatii*	–	300 bp	–

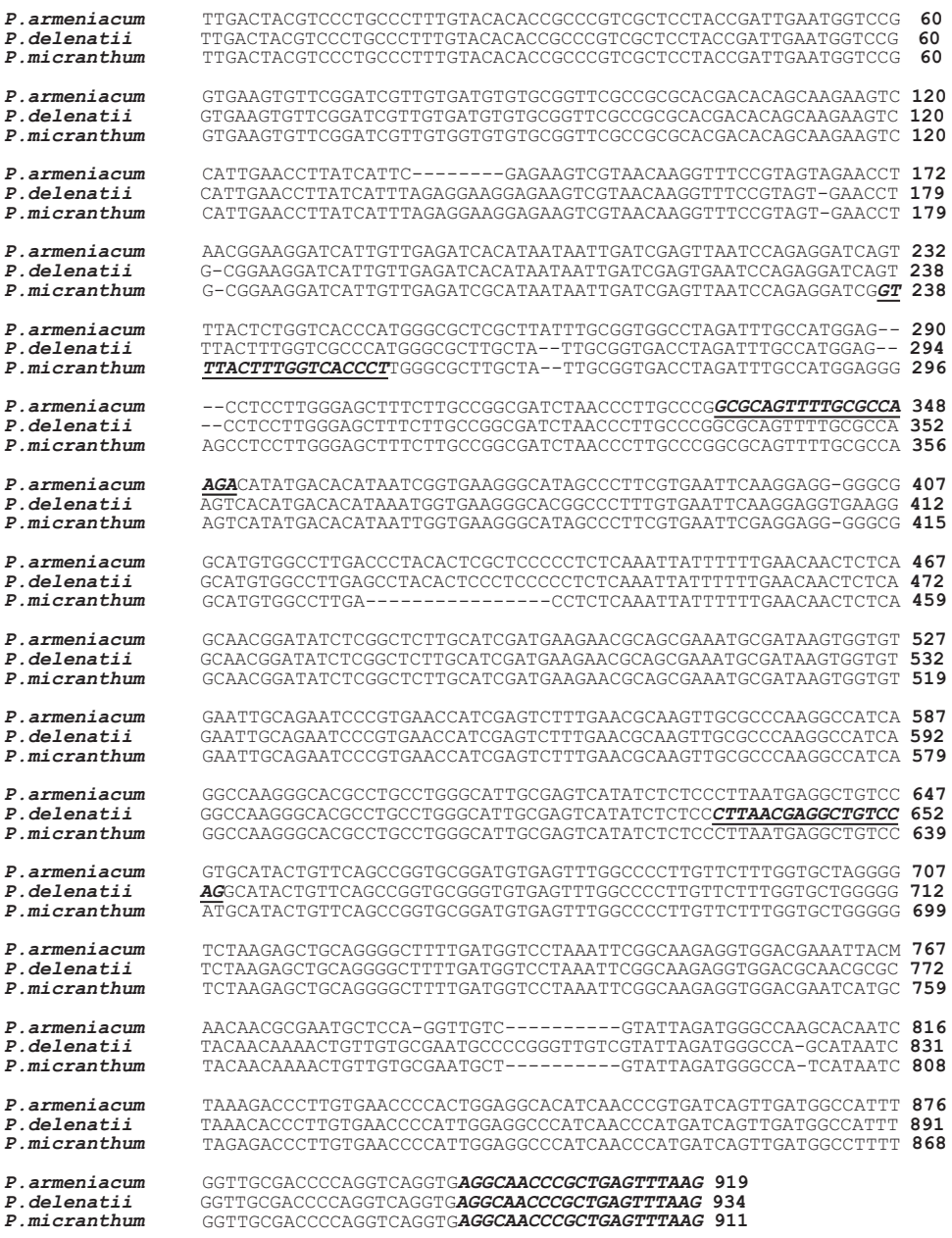

Fig. 2. Sequences comparison of the rDNA-ITS regions of *P. armeniacum*, *P. micranthum* and *P. delenatii*. The underlined italics parts indicate the sequences used for designing forward SCAR primers. The non-underlined italics parts indicate the sequences used for designing reverse SCAR primer. The hyphens indicate the missing nucleotides.

ber enable easy amplification of ITS region (Dubouzet and Shinoda, 1999). These advantages have made rDNA-ITS region as preferred choice for molecular typing. Thus, many studies in recent years have employed ITS sequences as genetic markers for various species (Zhang et al., 2007; Choo et al., 2009; Gulbitti-Onarici et al., 2009). In this study, the sequence of rDNA-ITS region and SCAR (sequence characterized amplified regions) markers were used as genetic markers for distinguishing *P. armeniacum, P. micranthum*, *P. delenatii* and their hybrid progenies.

To develop the SCAR markers for the identification of tested *Paphiopedilum* species, the rDNA-ITS regions in 31 *Paphiopedilum* species were amplified using Pap-18SF and Pap-ITS2R primers (Table 1). An 890 bp DNA fragment was detectable in all the tested *Paphiopedilum* species (Table 2). These rDNA-ITS fragments collected from the genomic DNA samples of *P. armeniacum*, *P. micranthum* and *P. delenatii* were sequenced (Fig. 2). The identified nucleotide sequences for the tested three species were similar to that of the sequences listed in GenBank (http://www.ncbi.nlm.nih.gov/). The identified sequences were further compared to each other to find species-specific primer regions. As a result, the species-specific regions were obtained by comparing three entire rDNA-ITS sequences (Fig. 2). These specifics-specific regions were used for developing species-specific SCAR markers.

As shown in Table 1, three SCAR primers SCAR-600armF/Pap-ITS2R, SCAR-300delF/Pap-ITS2R and SCAR-700micF/Pap-ITS2R were developed for discriminating *P. armeniacum*, *P. micranthum*, *P. delenatii* and their respective hybrids. SCAR-600armF/Pap-ITS2R gave a 600 bp product only with *P. armeniacum* and its hybrid progenies (Tables 2 and 3). On the other hand, SCAR-300delF/Pap-ITS2R gave a 300 bp product only with genomic DNA samples of *P. delenatii* and its hybrid progenies (Tables 2 and 3), while, only for *P. micranthum* and its hybrid progenies, SCAR-700micF/Pap-ITS2R gave a 700 bp product (Tables 2 and 3). These results indicate that

Fig. 3. Agarose gel of the PCR products using multiplex-PCR amplification of SCAR primer pairs SCAR-600armF/SCAR-300delF/SCAR-700micF/Pap-ITS2R, which amplify a single band specifically for *P. armeniacum* (600 bp), *P. delenatii* (300 bp) and *P. micranthum* (700 bp) among the tested 32 species, respectively.

the developed three SCAR markers serve the purpose of distinguishing *P. armeniacum*, *P. micranthum*, *P. delenatii* and their hybrid progenies.

In the present work, primers SCAR-600armF/Pap-ITS2R, SCAR-300delF/Pap-ITS2R and SCAR-700micF/Pap-ITS2R were also used simultaneously in multiplex-PCR amplification to improve the application of the designed SCAR markers. The amplification with combination of these three SCAR primers produced three clearly amplified PCR products, one for *P. armeniacum* at 600 bp, one for *P. delenatii* at 300 bp, and one for *P. micranthum* at 700 bp (Fig. 3). This multiplex-PCR amplification also efficiently produced similar patterns of DNA fragments enough to discriminate the hybrid progenies of *P. armeniacum*, *P. micranthum* and *P. delenatii* (Fig. 4). For instance, only a 600 bp fragment was obtained from the genomic DNA sample of *P. armeniacum* × *P. vietnamensei*. Similar 600 bp fragment was also found for the genomic DNA sample of *P. primulium* × *P. armeniacum* (Fig. 4). However, both 300 bp and 700 bp fragments were exhibited by the genomic DNA sample of *P. delenatii* × *P. micranthum* (Fig. 4). On the other hand, both 300 bp and 600 bp fragments were enhibited by *P. delenatii* × *P. armeniacum* (Fig. 4). These results clearly indicate that the resulting multi-species discrimination SCAR markers are effective for the

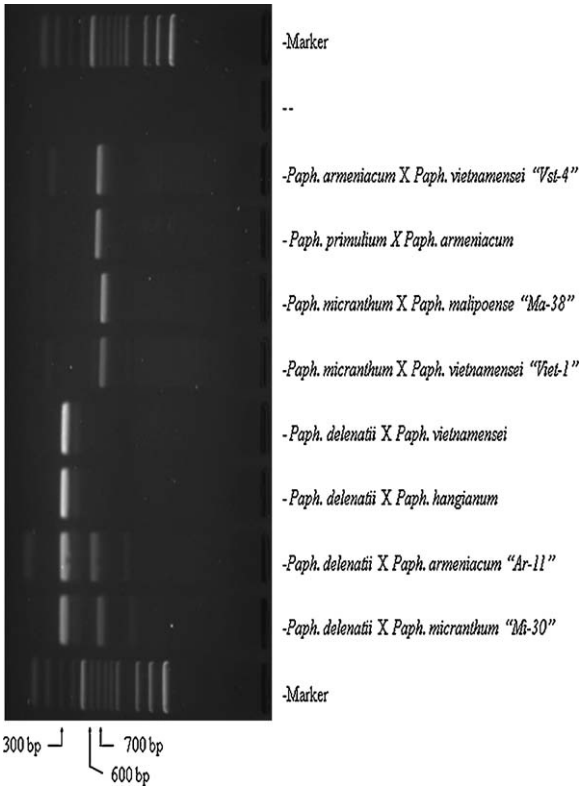

Fig. 4. Agarose gel of the PCR products using multiples-PCR amplification of primer pairs SCAR-600armF/SCAR-300delF/SCAR-700micF/Pap-ITS2R, which amplify one or two bands specifically for the tested hybrids of *P. armeniacum* (600 bp), *P. delenatii* (300 bp) and *P. micranthum* (700 bp), depending on the parent lines used.

identification of these three *Paphiopedilum* species and their hybrid progenies.

From the marker-assisted selection point of view, a reliable, fast, and simple PCR assay to identify the presence of species or variety differences is highly desirable. In this study, the rDNA-ITS sequence of three *Paphiopedilum* species including *P. armeniacum*, *P. micranthum* and *P. delenatii* were analyzed and compared. Three SCAR primers SCAR-600armF/Pap-ITS2R, SCAR-300delF/Pap-ITS2R and SCAR-700micF/Pap-ITS2R were further developed to distinguish these three species and their hybrid progenies. Additionally, these three SCAR markers were also used simultaneously in multiples-PCR amplification to improve the application of the SCAR markers, and the results confirmed that the combination of these markers is also capable of distinguishing each species and their hybrid progenies from closely related *Paphiopedilum* species. Thus, the designed SCAR markers can be used to test the species or hybrids origin of morphologically ambiguous individuals.

Acknowledgements

This study is supported in part by a grant from the Council of Agriculture of the Executive Yuan for financial support. We would like to thank Sin-Jhih Syong (Hung Shen Orchid Farm, http://dear.smartweb.tw) and Cheng-Zhong Chen (Mainshow Orchids, http://www.mainshow,com.tw) for providing the tested slipper materials.

References

Barbara, T., Palma-Silva, C., Paggi, G.M., Bered, F., Fay, M.F., Lexer, C., 2007. Cross-species transfer of nuclear microsatellite markers: potential and limitations. Mol. Ecol. 16, 3759–3767.

Choo, B.K., Moon, B.C., Ji, Y., Kim, B.B., Choi, G., Yoon, T., Kim, H.K., 2009. Development of SCAR markers for the discrimination of three species of medicinal plants, *Angelica decursiva* (*Peucedanum decursivum*) *Peucedanum praeruptorum* and *Anthricus sylvestris*, based on the internal transcribed spacer (ITS) sequence and random amplified polymorphic DNA (RAPD). Biol. Pharm. Bull. 32, 24–30.

Chung, S.Y., Choi, S.H., Kim, M.J., Yoon, K.E., Lee, G.P., Lee, J.S., Ryu, K.H., 2006. Genetic relationship and differentiation of *Paphiopedilum* and *Phragmepedium* based on RAPD analysis. Sci. Hortic. 109, 153–159.

Cox, A.V., Pridgeon, A.M., Albert, V.A., Chase, M.W., 1997. Phylogenetics of the slipper orchids (*Cypripedioideae Orchidaceae*): nuclear rDNA ITS sequences. J. Plant Syst. Evol. 208, 197–223.

Dubouzet, J.G., Shinoda, K., 1999. Relationships among old and new world *Alliums* according to ITS DNA sequence analysis. Theor. Appl. Genet. 98, 422–433.

Gulbitti-Onarici, S., Sancak, C., Sumer, S., Ozcan, S., 2009. Phylogenetic relationships of some wild wheat species based on the internal transcribled spacer sequences of nrDNA. Curr. Sci. 96, 794–800.

Hussain, Z., Tyagi, R.K., Sharma, R., Agrawal, A., 2008. Genetic diversity in *in vitro*-conserved germplasm of *Curcuma* L. as revealed by RAPD markers. Biol. Plant 52, 627–633.

Kumar, P., Gupta, V.K., Misra, A.K., Modi, D.R., Pandey, B.K., 2009. Potential of molecular markers in plant biotechnology. Plant Omics J. 2, 141–162.

Liu, Z., Liu, K., Chen, L., Lei, S., Li, L., Shi, X., Huang, L., 2006. Conservation ecology of endangered species *Paphiopedilum armeniacum* (Orchidaceae). Acta Ecol. Sinica 26, 2791–2800.

Rodrigues, K.F., Kumar, S.V., 2009. Isolation and characterization of 24 microsatellite loci in *Paphiopedilum rothschildianum*, and endangered slipper orchid. Conserv. Genet. 10, 127–130.

Soltis, D., Soltis, P.S., 2000. Contributions of plant molecular systematics to studies of molecular evolution. Plant Mol. Biol. 42, 45–75.

Zhang, Y.-B., Shaw, P.-C., Sze, C.-W., Wang, Z.-T., Tong, Y., 2007. Molecular authentication of Chinese herbal materials. J. Food Drug Anal. 15, 1–9.