Agr. Nat. Resour. 53 (2019) 340–347

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Genetic relationship of orchids in the Calanthe group based on sequence-related amplified polymorphism markers and development of sequence-characterized amplified regions markers for some genus/species identification

Surapa Nutthapornnitchakul[a], Surin Peyachoknagul[a,b], Pattamon Sangin[a,b], Anupan Kongbungkerd[a,b], Tassanai Punjansing[c] and Maliwan Nakkuntod[a,b,*]

[a] *Biotechnology Program, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand.*

[b] *Department of Biology, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand.*

[c] *Department of Biology, Faculty of Science, Udon Thani Rajaphat University, Udon Thani 41000, Thailand.*

Article Info

Article history:
Received 28 May 2018
Revised 2 July 2018
Accepted 11 July 2018
Available online 30 August 2019

Keywords:
Calanthe,
Cephalantheropsis,
Phaius,
SCAR markers,
SRAP markers

Abstract

The Calanthe comprises a group of terrestrial orchid species known for their attractive flowers consisting of three genera (*Calanthe*, *Phaius* and *Cephalantheropsis*) which share many similar characteristics causing difficulty in identification. Here, the genetic relationship of the Calanthe group was studied using sequence-related amplified polymorphism (SRAP) markers. Using 18 primer pairs, 565 fragments were generated in which 562 were polymorphic (99.45%) indicating a high level of polymorphism in the Calanthe. A dendrogram was constructed using the unweighted pair group method with arithmetic mean method. Calanthe orchids were separated into five clades consisting of three groups of the genus *Calanthe*, one *Phaius* group and one consisting of *Calanthe* and *Cephalantheropsis* species. Overall clusters were in agreement with their morphologies and other reports using DNA sequences of the chloroplast genome, indicating the effectiveness of the SRAP markers; however, *C. rubens* var. *alba* was not closely related to *C. rubens*, suggesting a possible identification error. Sequence characterized amplified regions (SCAR) primers for *P. flavus* and *C. herbacea* species identification were also developed. The results showed that PFLF/PFLR primers could be used to amplify the specific fragment of 240 bp only in *P. flavus*. CHEF/CHER primers were used to amplify the fragment of 430 bp in three species (*C. masuca*, *C. triplicata* and *C. herbacea*) which were in the same clade. SCAR markers were successfully developed for *P. flavus* identification and detection of the *C. masuca*-*C. triplicata*-*C. herbacea* clade.

* Corresponding authors.
E-mail address: lotharmali@yahoo.com (M. Nakkuntod)

https://doi.org/10.34044/j.anres.2019.53.4.03

Introduction

The Orchidaceae is one of the largest families of flowering plants with five subfamilies and about 870 genera (Swarts and Dixon, 2009). Some species in this family are threatened while others are endemic and some plant numbers have been reduced by human activities such as over-collection due to their ornamental and medicinal values (Qian et al., 2013). Orchids are also commercially traded in markets for their beautiful flowers and selected for breeding based on their favorable characteristics as they are extraordinary plants which can be crossed among different species or even among different genera producing viable hybrids which can then be propagated (De, 2017). Hence, it is very difficult to identify orchid species or parent tracking with unknown hybrids based on morphological traits (Freudenstein and Rasmussen, 1999).

Punjansing et al. (2017) discussed the Calanthe group in detail which is summarized in the following text. The Calanthe group is composed of three genera (*Calanthe* R. Br., *Phaius* Lour., and *Cephalantheropsis* Guillaumin). They are members of the family Orchidaceae, subfamily Epidendroideae which are terrestrial or epiphytic herbs having pseudobulbous and elongate-fusiform cane-like stems. Numbers of basal sheaths below the pseudobulb are few and mostly already partly or entirely decayed at flowering time. The orchid genus *Calanthe* R. Br. is widely distributed in Asia, Australia and the Pacific Ocean islands, with 260 species worldwide, but only 21 have been found in Thailand (Hotsunimi et al., 1989; Yoon, 1990). Strong points of the *Calanthe* genus are its prominent inflorescence having colorful and long-lasting florets which can be used for inter- or intra-specific breeding. There are 40 species of the genus *Phaius* Lour. distributed in Asia, the Malay Archipelago and the Philippines, including five species in Thailand (Kurzweil, 2010). Based on morphological data, they can be divided into two types of bract (caducous or persistent). Most of the species in this group have large flowers with various colors. The genus *Cephalantheropsis* Guillaumin comprises five species from India to the Maluku Islands and northward as far as China, Taiwan, the Philippines and Japan while only two species have been found in Thailand (Kurzweil, 2010; Pedersen et al., 2014). All orchids in the Calanthe group have abundant shared characteristics such as distichous plicate leaves, basal and lateral inflorescence, resupinate flowers with free sepals and petals, spurred lips and eight pollinia. Because of these shared characteristics, morphological classification is ambiguous with poor taxonomic classification (Zhai et al., 2014).

Molecular markers are very important and commonly used in genetic map construction, genetic diversity analysis and marker-assisted selection in plant breeding (Li et al., 2013). In addition, various types of molecular markers have been developed and applied in agricultural crop breeding (Nadeem et al., 2017). Several molecular marker technologies are available for studying genetic relationships in plants such as random amplified polymorphic DNA or RAPD (Williams et al., 1990), Amplified fragment length polymorphism or AFLP (Vos et al., 1995) and sequence-related amplified polymorphism or SRAP (Li and Quiros, 2001).

The SRAP marker technique was developed by Li and Quiros (2001) to construct a genetic map in *Brassica oleracea* L. This marker has since been adapted for various purposes such as germplasm identification, map construction, gene tagging, genomics and cDNA fingerprinting and map-based cloning (Amar et al., 2011). SRAP markers have been used in many plants including two species of *Dendrobium* (Ding et al., 2008; Cai et al., 2011), saffron (Babaei et al., 2014), *Pinus* species (Xie et al., 2015) and water melon (Yagcioglu et al., 2016). These studies demonstrated that the SRAP marker technique is a simple, efficient and reliable tool for studying genetic relationships and diversity. Furthermore, SRAP markers could be converted to SCAR (sequence-characterized amplified regions) markers that provide higher specificity, more reproducibility and easier operation (Ding et al., 2010). The SCAR marker technique was developed by Paran and Michelmore (1993) to study downy mildew resistance genes in lettuce. SCAR markers are effective because they can be used for various benefits including detection from raw materials and processed products of *Curcuma longa* L. (Dhanya et al., 2011) and saffron (Torelli et al., 2014). Moreover, SCAR markers have been used for identification and genetic study in various commercial and medicinal plants such as *Paphiopedilum* spp. and their hybrids (Sun et al., 2011), cluster bean (Sharma et al., 2014), *Hypsizygus marmoreus* (Peck) Bigel. (Sun et al., 2016), papaya (Liao et al., 2017) and *Zea mays* Linn (Roy et al., 2018). Thus, as both SRAP and SCAR are powerful molecular markers, the genetic relationship in Calanthe orchids was investigated using SRAP markers and converting some polymorphic bands to SCAR markers for identification of orchid species.

Materials and Methods

Plant materials

Twenty orchid species in the Calanthe group comprising 13 species of *Calanthe*, five species of *Phaius* and two species of *Cephalantheropsis* were collected from locations in northern Thailand consisting of: the Queen Sirikit Botanic Garden, Chiang Mai; Ban Romklao Botanical Garden, Phitsanulok; Khunchuyjang Orchid Farm, Chiang Mai; and from local markets in Loei and Phetchabun provinces.

DNA extraction

Genomic DNA samples were extracted from the leaves of the 20 Calanthe orchid species using the CTAB method modified from Doyle and Doyle (1987). DNA integrity was checked using 0.8% agarose gel electrophoresis and the optical density was measured using a microplate reader (BioTek, Winooski, VT,, USA). After quantification, the DNA was diluted with distilled water to a working concentration of 100 ng/µl for PCR analysis.

Screening of SRAP primers

One hundred primer pairs of M and E primer sets (Table 1) modified from Li and Quiros (2001) were screened in three representative samples of each genus (*C. densiflora*, *P. flavus* and *Cep. obcorcata*). Suitable primer combinations were selected that could amplify in all three samples having polymorphic bands.

SRAP analysis

Suitable primers were used to amplify all species of the Calanthe group. PCR amplification was carried out with an initial denaturation step at 94°C for 5 min, followed by 5 cycles of denaturing at 94°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 1 min, followed by 35 cycles of high annealing temperature at 52°C. A final extension step was performed at 72°C for 5 min. PCR products were separated using 2.5% agarose gel electrophoresis in Tris-borate-EDTA buffer at 80V for 5 hours and stained with ethidium bromide.

Phylogenetic analysis

SRAP profiles were scored in terms of a binary data matrix indicating presence (code 1) or absence (code 0). Only strong and clearly distinguished bands were used for phylogenetic analysis. Percentages of polymorphic bands were calculated and performance of the markers was measured using polymorphic information content (PIC) values. The PIC value was calculated according to Hardy-Weinberg equilibrium as $PIC_i = 2f_i(1\text{-}f_i)$, where f_i is the frequency of the amplified allele (band present) and $1\text{-}f_i$ is the frequency of the null allele (Roldan-Ruiz et al., 2000). A dendrogram of SRAP fingerprints was generated using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) and the NTSYSpc software version 2.20e (Rohlf, 2000); genetic similarities were computed using Jaccard's coefficient (Jaccard, 1908).

Development of SCAR markers

Specific bands were excised from agarose gel and DNA was extracted using PureDireX PCR Clean-up & Gel Extraction Kit (BioHelix, Keelung, Taiwan). Purified DNA fragments were ligated into pTZ57R/T vector (Thermo Scientific; Waltham, MA, USA) and transformed into *Escherichia coli* DH5α competent cells. Recombinant clones were selected on the basis of blue and white screening and cloned DNA fragments were sequenced by Macrogen Inc.; Seoul, South Korea. Sequences were compared using BLAST in the GenBank database (http://www.ncbi.nlm.nih.gov). SCAR primers were manually designed according to the DNA sequences synthesized by Macrogen Inc. (Seoul, South Korea) and tested in 20 samples of the Calanthe group and three other species (*Epipactis flava*, *Triticum aestivum* cv. Capo and *Dendrobium*).

Results and Discussion

SRAP primer screening and analysis

Out of the 100 primer combination screenings, only 18 primer pairs were selected. Among the 82 primers remaining, 14 could not amplify in Calanthe orchids, 17 could amplify in some samples, 51 could amplify in all samples, 9 provided monomorphic bands and 42 gave unclear results. Therefore, these primers were screened out.

The 18 selected SRAP primer pairs generated 565 clear and reproducible fragments, with an average of 31.39 bands/primer ranging in size from 100 to 1,500 bp, of which 562 were polymorphic and averaged 99.45% polymorphism (Table 2). The results of each primer showed a higher percentage of polymorphism than other reports because this study comprised 20 species from three different genera, while other studies were only conducted within the genus and species levels. For example, the genera *Dendrobium* (Feng et al., 2013), *Pinus* (Xie et al., 2015), and *Portulaca* (Jia et al., 2017) were studied at the genetic level, whereas, *Cuminum cyminum* species (Bhatt et al., 2017) and *Brassica oleracea* were investigated at the species levels. All samples in both taxonomic levels demonstrated close relationships. Thus, this is the first report to use SRAP markers at the higher-generic level as a wider range of taxonomic hierarchy. The PIC values from all 562 loci ranged from 0.00 to 0.50 with an average of 0.15. The PIC value is another option for detecting polymorphism in markers. Here, their values were in the medium range and characteristics of dominant markers with two alleles.

Table 1 Names and sequences of sequence-related amplified polymorphism primers used

Primer name	Primer sequence (5′-3′)	Primer name	Primer sequence (5′-3′)
M1	TGA GTC CAA ACC GGA AA	E1	GAC TGC GTA CGA ATT AAC
M2	TGA GTC CAA ACC GGA AG	E2	GAC TGC GTA CGA ATT AAT
M3	TGA GTC CAA ACC GGA AC	E3	GAC TGC GTA CGA ATT GAC
M4	TGA GTC CAA ACC GGA AT	E4	GAC TGC GTA CGA ATT GCA
M5	TGA GTC CAA ACC GGA GC	E5	GAC TGC GTA CGA ATT CAA
M6	TGA GTC CAA ACC GGA CA	E6	GAC TGC GTA CGA ATT CAG
M7	TGA GTC CAA ACC GGA CC	E7	GAC TGC GTA CGA ATT CAC
M8	TGA GTC CAA ACC GGA TA	E8	GAC TGC GTA CGA ATT CGT
M9	TGA GTC CAA ACC GGT AG	E9	GAC TGC GTA CGA ATT TGA
M10	TGA GTC CAA ACC GGT CA	E10	GAC TGC GTA CGA ATT TGC

Table 2 Total bands of amplified fragments and polymorphic bands generated by polymerase chain reaction- sequence-related amplified polymorphism of the Calanthe group

Primer combination	Total band	Polymorphic band	Polymorphic band (%)	Range of polymorphic information content and (mean)
M1E7	33	33	100	0.05−0.50 (0.18)
M2E1	44	44	100	0.05−0.50 (0.12)
M2E2	25	25	100	0.05−0.35 (0.15)
M2E4	24	24	100	0.05−0.27 (0.13)
M2E5	36	36	100	0.05−0.41 (0.16)
M2E6	35	35	100	0.05−0.46 (0.16)
M2E7	32	30	93.75	0.00−0.38 (0.13)
M2E9	39	39	100	0.05−0.44 (0.14)
M2E10	28	28	100	0.05−0.38 (0.17)
M3E6	28	28	100	0.05−0.31 (0.14)
M3E9	29	29	100	0.05−0.35 (0.15)
M4E3	32	32	100	0.05−0.41 (0.16)
M5E2	27	26	96.30	0.00−0.27 (0.16)
M6E10	35	35	100	0.05−0.47 (0.16)
M9E2	40	40	100	0.05−0.46 (0.16)
M9E4	23	23	100	0.05−0.50 (0.18)
M9E5	26	26	100	0.05−0.44 (0.20)
M10E1	29	29	100	0.05−0.44 (0.15)
Total	565	562	-	-
Average	31.39	31.22	99.45	0.15

The data matrix of genetic similarities showing the relationship in the Calanthe group is presented in Fig. 1. Genetic similarity among the Calanthe group ranged from 0.06 to 0.59 (average 0.149), and was low compared to other plants (Jehan et al., 2014). The genetic similarity coefficient in the genus *Calanthe* ranged from 0.06 (between *C. cardioglossa* and *C. masuca*) to 0.40 (between *C. rubens* and *C. rosea*), and from 0.12 (between *P. flavus* and *P. tankervilleae*) to 0.59 (between *P. indochinensis* and *P. mishmensis*) within the genus *Phaius*, and was 0.33 between the two species in the genus *Cephalantheropsis*. Average genetic similarities within the genera *Calanthe*, *Phaius*, and *Cephalantheropsis* were 0.148, 0.259, and 0.33, respectively, which corresponded well with the number of orchid species in each genus.

	C. cardioglossa	*C. clavata*	*C. densiflora*	*C. lyroglossa*	*C. masuca*	*C. pulchra*	*C. rosea*	*C. rubens*	*C. succedanea*	*C. triplicata*	*C. vestita*	*C. herbacea*	*C. rubens* var. *alba*	*P. flavus*	*P. indochinensis*	*P. mishmensis*	*P. tankervilleae*	*P. tankervilleae* var. *alba*	*Cep. obcordata*	*Cep. longipes*
C. cardioglossa	1.00																			
C. clavata	0.12	1.00																		
C. densiflora	0.11	0.28	1.00																	
C. lyroglossa	0.09	0.18	0.21	1.00																
C. masuca	0.06	0.10	0.09	0.13	1.00															
C. pulchra	0.07	0.14	0.21	0.40	0.10	1.00														
C. rosea	0.11	0.13	0.16	0.11	0.12	0.09	1.00													
C. rubens	0.15	0.15	0.12	0.14	0.10	0.09	0.37	1.00												
C. succedanea	0.16	0.13	0.12	0.13	0.10	0.09	0.20	0.26	1.00											
C. triplicata	0.08	0.12	0.14	0.10	0.24	0.09	0.16	0.15	0.16	1.00										
C. vestita	0.11	0.10	0.08	0.14	0.13	0.10	0.22	0.35	0.23	0.14	1.00									
C. herbacea	0.11	0.09	0.11	0.08	0.26	0.08	0.13	0.12	0.11	0.32	0.13	1.00								
C. rubens var. *alba*	0.10	0.15	0.14	0.11	0.10	0.13	0.34	0.20	0.17	0.16	0.16	0.10	1.00							
P. flavus	0.10	0.11	0.15	0.13	0.11	0.11	0.18	0.12	0.16	0.14	0.10	0.10	0.19	1.00						
P. indochinensis	0.10	0.15	0.18	0.16	0.11	0.16	0.17	0.13	0.13	0.12	0.11	0.12	0.13	0.23	1.00					
P. mishmensis	0.13	0.15	0.17	0.19	0.10	0.16	0.18	0.11	0.14	0.14	0.11	0.12	0.15	0.24	0.59	1.00				
P. tankervilleae	0.08	0.19	0.15	0.14	0.10	0.11	0.16	0.10	0.11	0.13	0.13	0.12	0.19	0.13	0.18	0.18	1.00			
P. tankervilleae var. *alba*	0.09	0.17	0.19	0.16	0.08	0.13	0.15	0.11	0.13	0.15	0.13	0.11	0.16	0.12	0.17	0.18	0.57	1.00		
Cep. obcordata	0.12	0.20	0.25	0.30	0.09	0.15	0.18	0.12	0.15	0.14	0.09	0.14	0.17	0.15	0.17	0.18	0.17	0.17	1.00	
Cep. longipes	0.13	0.15	0.14	0.20	0.11	0.11	0.13	0.13	0.13	0.11	0.09	0.13	0.12	0.10	0.12	0.14	0.11	0.10	0.33	1.00

Fig. 1 Similarity index of orchids in the Calanthe group

Genetic relationship analysis

Data obtained from the SRAP analysis of the 20 species/varieties of Calanthe orchids were subjected to cluster analysis. The UPGMA dendrogram is shown in Fig. 2. Samples were divided into five clades at the similarity coefficient of 0.158. The first clade consisted of three species of the genus *Calanthe*: *C. masuca*, *C. triplicata* and *C. herbacea*. These species present important characteristics such as an unnoticeable pseudobulb and a downward pointing labellum spur (Kurzweil, 2010; Pedersen et al., 2014). In addition, Zhai et al. (2014) placed orchids in this clade under the genus *Calanthe* according to data on the chloroplast DNA sequence. Five samples of the genus *Calanthe* (*C. rosea*, *C. rubens*, *C. rubens* var. *alba*, *C. vestita* and *C. succedanea*) were placed in the second clade, showing the same morphological characters of a palpable pseudobulb, foliage leaves presented once a year and mostly not during flowering (Pedersen et al., 2014). The results from the dendrogram indicated that *C. rubens* var. *alba* was not closely related to *C. rubens*; its similarity index was most similar to *C. rosea* suggesting an identification error. This sample might be another species and not *C. rubens* var. *alba*; however, it should remain classified as *Calanthe* sp. until clarification is undertaken from investigation using other methods such as DNA barcoding. The third clade consisted of five accessions of *Phaius* (*P. flavus*, *P. indochinensis*, *P. mishmensis*, *P. tankervilleae* and *P. tankervilleae* var. *alba*) which have similar habitats and a shared stem or pseudobulbous and plicate leaf characteristics with the genus *Calanthe*. Thus, *Phaius* was grouped among the *Calanthe* species. *P. indochinensis*, formerly classified as *P. mishmensis* showed the highest similarity to *P. mishmensis* with the shared characteristic of inflorescence on the lateral side of the cane-like stem (Pedersen et al., 2014). Based on the similarity index, *P. tankervilleae* var. *alba* was different from *P. tankervilleae*. In the future, *P. tankervilleae* var. *alba* might be classified as another species. The fourth clade comprised two genera: *Calanthe* (*C. clavata*, *C. densiflora*, *C. lyroglossa*, *C. pulchra*) and *Cephalantheropsis* (*Cep. obcordata* and *Cep. longipes*). Zhai et al. (2014) reported *Cephalantheropsis* as a sister group of the *Calanthe* sectcalled the *Styloglossum* (*C. clavata*, *C. densiflora*, *C. lyroglossa*) clade. Chase et al. (2015) also reported *Cephalantheropsis* as a sister clade of *C. clavata* and *C. densiflora*. The fifth clade contained a single species (*C. cardioglossa*) at the base of the dendrogram separated from the Calanthe group. This species has a strong vein on the mid-lobe (Kurzweil, 2010) which is a unique characteristic. However, further study using other techniques is recommended because this species lacks sufficient data for classification.

In summary, the Calanthe group is a complex taxon with genetic relationships of a monophyletic group. The *Calanthe* genus has been divided into many sections (Zhai et al., 2014) as well as in this study. Most amplified SRAP fragments were from the nuclear genome (Li and Quiros, 2001) which provided a good representative for evolutionary study. The results also corresponded well with barcoding data of chloroplast DNA (Zhai et al., 2014), indicating the effectiveness of the SRAP method in a wide range of species.

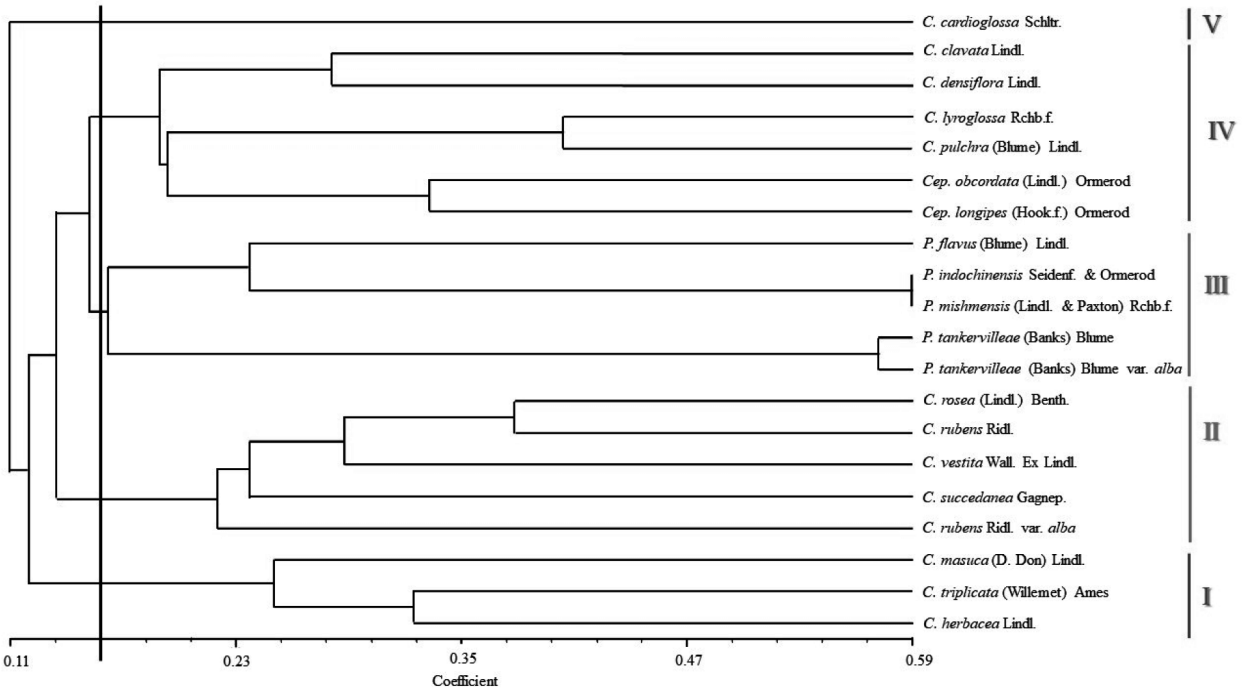

Fig. 2 Unweighted pair group method with arithmetic mean (UPGMA) dendrogram showing genetic relationship of the Calanthe group based on Jaccard's coefficient of sequence-related amplified polymorphism markers

Development of SCAR markers

Specific fragments from two species, *P. flavus* and *C. herbacea*, were selected for development of SCAR markers. Both species have beautiful, medium-sized flowers and are suitable for breeding programs and commercial development. Therefore, specific bands were cloned and sequenced (Fig. 3). The Blast results showed that these two sequences were homologous to uncharacterized mRNA sequences from *Dendrobium catenatum* and *Erycina pusilla*, respectively, in the GenBank database, indicating that SRAP markers had high potential for amplification from the open reading frame as reported by Li and Quiros (2001). The primers PFLF (5'- GAG ACC ATT TGT CAA GGC C-3') and PFLR (5'- C AGA ATA TTG GAA GAC TCA GGC-3') specific to *P. flavus* were designed and used to amplify the fragment of 240 bp for *P. flavus* detection. Three other plant species were also tested with this primer and no band could be detected (Fig. 4A) indicating its specificity to *P. flavus*. Moreover, this primer could amplify in both samples of *P. flavus* collected from different clones (Fig. 4B). Other specific primers from the *C. herbacea* fragment were designed as CHEF1 (5'- CCA ATT CAC ACA AAT CGA GTC G -3') and CHER (5'- GAA GGT GTT TGT CCT TAA CGG -3'). This marker was used to amplify DNA fragments from 20 samples of the Calanthe group. However, the SCAR primer from *C. herbacea* was not only specific to its species but could also amplify in the three species of the *Calanthe* clade I (*C. masuca*, *C. triplicata and C. herbacea*), giving similar-sized fragments of 430 bp and supporting the result from phylogenetic analysis (Fig. 4C). Therefore, SCAR markers may have additional specificity to species and selection which greatly assists in plant identification (Bhagyawant, 2016).

(A)

GGATAGACCCGTCAATGAATCTGGTTGGAATTTAGGAGACCA
TTTGTCAAGGCCAATAATATGCCCCGCAATTTGCCATGGCCC
CCCCATCAGGACAGCATCTCTAGCTTCAAAGCTATCAAAGAG
GCATATAAAGCAGTCAGTTTCGATCAAAACTGTACTGAACTC
GCCAAAACAGCTCCACCGGCGTTGCAGTTCAGCATCAAGGA
TGGCAAAAGGCAACTTCTTCCCAATGAGACGTCCAACCAAA
GCGCGCCTGAGTCTTCCAATATTCTGGTTTAAAGCAGTTTCA
TCAAGAGCAACAGTTTTTCCATCCAGAGAGATCGTCCCCAA
CTCTTCCTT

(B)

ATTACTTTAGCCGCGGGAGGATCTCCAATTCACACAAATCGAGTCGTAG
TTCTTCCTTCCCTACATAGGCTTCCTTCTTCATACACTGAATCCATTTTTC
AGAAAACATTGAAACTGGAAAGTAGATTTGTGCTCTTCATTGGCGGCTT
GGCTTATACGCCTCTGACCGATTCTGCTTTTGAAACATCATCGATTCTCG
CTCTTATTCTCGGCAGCTGCAGGTACTTGTTTGTGAAAAAACAAACATA
GGTGAAAAAATATCCCCCATCTGAAGTAAGCAGTAGACCGGCGTGGCA
CAAAAGATCGTCATGGCATATGTGATGTGTGTACGGACATTGAAGAAGA
TCACTGTCAGGTACAGGTTTCCTATACTGAGGATGGTAATATATAGGCGG
TTTTTCAAAATTATGTGCTGGTAGTGACGGCATCAGATAAGAATCCGTTA
AGGACAAACACCTTCAAGAAAACATTCAAAGTAATGCATGGT

Fig. 3 DNA sequences of fragments from: (A) *P. flavus*, (B) *C. herbacea*. Sequences or positions of primers are underlined

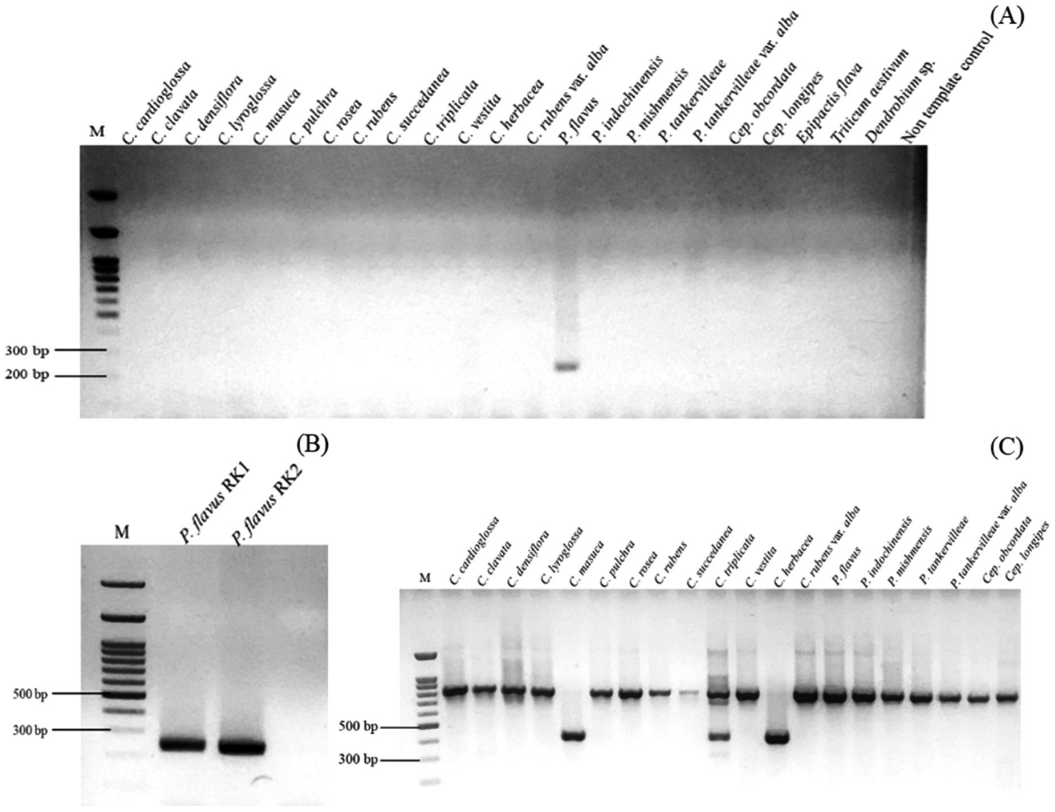

Fig. 4 DNA fragments amplified using sequence-characterized amplified regions primers: (A and B) *P. flavus,* (C) *C. herbacea*

Ethics Statement

Biosafety and all experimental procedures were approved by the Biosafety Committee Naresuan University (Approval no. NUIBC GM 59-09-49 (59-69)).

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

The research was supported by grants from the National Research Council of Thailand (NRCT), Naresuan in the 2017 academic year (Research number: R2560B128) and a Science Achievement Scholarship of Thailand (SAST) from the Office of the Higher Education Commission, Thailand to the first author. The authors thank Dr. Rerngwit Boonyom (Faculty of Allied Health Sciences, Naresuan University) for bacterial culture and Mr. Waroon Suwankitti for help and suggestions. Finally, thanks are extended to the Queen Sirikit Botanic Garden, Khunchuyjang Orchid Farm and Ban Romklao Botanical Garden for providing the orchid samples.

References

Amar, M.H., Biswas, M.K., Zhang, Z., Guo W. 2011. Exploitation of SSR, SRAP and CAPS-SNP markers for genetic diversity of *Citrus* germplasm collection. Sci. Hortic. 128: 220–227.

Babaei, S., Talebi, M., Bahar, M., Zeinali, H. 2014. Analysis of genetic diversity among saffron (*Crocus sativus*) accessions from different regions of Iran as revealed by SRAP markers. Sci. Hortic. 171: 27–31.

Bhagyawant, S.S. 2016. RAPD-SCAR markers: an interface tool for authentication of traits. J. Biosci. Med. 4: 1–9.

Bhatt, J., Kumar, S., Patel, S., Solanki, R. 2017. Sequence-related amplified polymorphism (SRAP) markers based genetic diversity analysis of cumin genotypes. Ann. Agrar. Sci. 15: 434–438.

Cai, X., Feng, Z., Zhang, X., Xu, W., Hou, B., Ding, X. 2011. Genetic diversity and population structure of an endangered orchid (*Dendrobium loddigesii* Rolfe) from China revealed by SRAP markers. Sci. Hortic. 129: 877–881.

Chase, M.W., Cameron, K.M., Freudenstein, J.V., Pridgeon, A.M., Salazar, G.A., van den Berg, C., Schuiteman, A. 2015. An updated classification of Orchidaceae. Bot. J. Linn. Soc. 177: 151–174.

De, L.C. 2017. Improvement of ornamental plants - A review. Int. J. Hortic. 7: 180–204. doi: 10.5376/ijh.2017.07.0022.

Dhanya, K., Syamkumar, S., Siju, S., Sasikumar, B. 2011. Sequence characterized amplified region markers: A reliable tool for adulterant detection in turmeric powder. Food Res. Int. 44: 2889–2895.

Ding, G., Zhang, D., Ding, X., Zhou, Q., Zhang, W., Li, X. 2008. Genetic variation and conservation of the endangered Chinese endemic herb *Dendrobium officinale* based on SRAP analysis. Plant Syst. Evol. 276: 149–156.

Ding, W., Cao, Z., Cao, L. 2010. Molecular analysis of grass carp (*Ctenopharyngodon idella*) by SRAP and SCAR molecular markers. Aquacult. Int. 18: 575–587.

Doyle, J.J., Doyle, J.L. 1987. A rapid isolation procedure from small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11–15.

Feng, S.G., Lu J.J., Gao, L., Liu, J.J., Wang, H.Z. 2013. Molecular phylogeny analysis and species identification of *Dendrobium* (Orchidaceae) in China. Biochem. Genet. 52: 127–36.

Freudenstein, J.V., Rasmussen, F.N. 1999. What does morphology tell us about orchid relationships? - A cladistic analysis. Amer. J. Bot. 86: 225–248.

Hotsunimi, T.R., Ogani, K., Hosika, A.Y., Yamazaki, N., Nitsuta, A., Yanagi, M.N. 1989. Useful plants of the world. Peongbu Publishing Company, Tokyo. Japan, pp. 190–191.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44: 223–270. [in French]

Jehan, T., Vashishtha, A., Yadav, S.R., Lakhanpaul, S. 2014. Genetic diversity and genetic relationships in Hyacinthaceae in India using RAPD and SRAP markers. Physiol. Mol. Biol. Plants. 20: 103–114.

Jia, S., Yan, Z., Wang, Y., Wei, Y., Xie, Z., Zhang, F. 2017. Genetic diversity and relatedness among ornamental purslane (*Portulaca* L.) accessions unraveled by SRAP markers. 3 Biotech. 7: 241. doi 10.1007/s13205-017-0881-8.

Kurzweil, H. 2010. A precursory study of the *Calanthe* group (Orchidaceae) in Thailand. Adansonia. 32: 57–107.

Li, G., Quiros, C.F. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in *Brassica*. Theor. Appl. Genet. 103: 455–461.

Li, G., McVetty, P.B.E., Quiros, C.F. 2013. SRAP Molecular Marker Technology in Plant Science. In: Anderse, S.B. (Ed.). Plant Breeding from Laboratories to Fields. IntechOpen. London, United Kingdom, pp. 23–43.

Liao, Z., Yu, Q., Ming, R. 2017. Development of male-specific markers and identification of sex reversal mutants in papaya. Euphytica. 213: 53. doi 10.1007/s10681-016-1806-z.

Nadeem, M., Nawaz, M., Shahid, M., et al. 2017. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. Biotechnol. Biotec. Eq. 32: 261–285.

Paran, I., Michelmore, R.W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85: 985–993.

Pedersen, H., Kurzweil, H., Suddee, S., et al. 2014. Flora of Thailand, Volume 12, Part 2: Orchidaceae 2 (Epidendroideae P.P: Neottieae, Tropideae, Nervilieae, Gastrodieae, Thaieae, Calypsoeae, Arethuseae, Collabieae, Cymbidieae). Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation. Bangkok, Thailand.

Punjansing, T., Chaichanachap, N., Sanitchon, J., Pinta, W., Nakkuntod, M. 2017. Genetic diversity of some species in the genus *Calanthe* analyzed by RAPD markers. Acta Hortic. 1167: 323–330.

Qian, X., Wang, C., Tian, M. 2013. Genetic diversity and population differentiation of *Calanthe tsoongiana*, a rare and endemic orchid in China. Int. J. Mol. Sci. 14: 20399–20413.

Rohlf, F.J. 2000. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.2. Exeter Software. Setauket. New York, NY, USA.

Roldan-Ruiz, I., Dendauw, J., Bockstaele, E.V., Depicker, A., Loose, M.D. 2000. AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). Mol. Breed. 6: 125–134.

Roy, N.S., Park, K., Lee, S., Im, M., Ramekar, R.V., Kim, N. 2018. Development of CACTA transposon derived SCAR markers and their use in population structure analysis in *Zea mays*. Genetica. 146: 1–12.

Sharma, P., Kumar, V., Raman, K.V., Tiwari, K. 2014. A set of SCAR markers in cluster bean (*Cyamopsis tetragonoloba* L. Taub) genotypes. Adv. Biosci. Biotechnol. 5, 131–141.

Sneath, P.H., Sokal, R.R. 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification, 1st ed. In: Freeman, W.H., San Francisco, CA, USA.

Sun, S., Feng, H., Chen, Z., Hu, K., Zhang, L. 2016. The first genetic linkage map of *Hypsizygus marmoreus* based on SCAR marker. Chiang Mai J. Sci. 43: 503–510.

Sun, Y.W., Liao, Y.J., Hung, Y.S., Chang, J.C., Sung, J.M. 2011. Development of ITS sequence based SCAR markers for discrimination of *Paphidopedilum armeniacum*, *Paphidopedilum micranthum*, *Paphidopedilum delenatii* and their hybrids. Sci. Hortic. 127: 405–410.

Swarts, N.D., Dixon, K.W. 2009. Terrestrial orchid conservation in the age of extinction. Ann. Bot. 104: 543–556.

Torelli, A., Marieschi, M., Bruni, R. 2014. Authentication of saffron (*Crocus sativus* L.) in different processed, retail products by means of SCAR markers. Food Control. 36: 126–131.

Vos, P., Hogers, R., Bleeker, M., et al. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407–4414.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 22: 6531–6535.

Xie, Q., Liu, Z., Wang, S., Li, Z. 2015. Genetic diversity and phylogenetic relationships among five endemic *Pinus* taxa (Pinaceae) of China as revealed by SRAP markers. Biochem. Syst. Ecol. 62: 115–120.

Yagcioglu, M., Gulsen, O., Solmaz, I., Yetisir, H., Sari, N. 2016. Genetic analyses of Turkish water melons based on SRAP markers. Turk. J. Agric. For. 40: 613–620.

Yoon, P.S. 1990. The wild plants of Korea. Nongwoo press. Seoul, Korea. pp. 52–53.

Zhai, J.W., Zhang, G.Q., Li, L., et al. 2014. A new phylogenetic analysis sheds new light on the relationships in the *Calanthe* alliance (Orchidaceae) in China. Mol. Phylogenet. Evol. 77: 216–222.