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Development of SCAR Markers for Species Identification of the Genus *Nepenthes* (Nepenthaceae)

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Abstract: *Nepenthes* species in Thailand, namely *N. mirabilis* Druce, *N. gracilis* Korth., *N. smilesii* Hemsl., *N. ampullaria* Jack and *N. kampotiana* Lecomte, were collected for development of Sequence Characterized Amplified Region (SCAR) marker, a genotype identification tool. Forty Random Amplified Polymorphic DNA (RAPD) primers were screened and three successful primers produced different banding patterns including five candidate species-specific markers. The candidate markers were cloned and sequenced. The marker sequences are 602, 379, 420, 473 and 1,017 bp for *N. mirabilis*, *N. gracilis*, *N. smilesii*, *N. ampullaria* and *N. kampotiana*, respectively. Then the sequences were used to design primers for development of a species-specific band being a SCAR marker, including Mir 1, Mir 2 and Mir 3 for *N. mirabilis*; Gra 1 and Gra 2 for *N. gracilis*; Smi 1, Smi 2 and Smi 3 for *N. smilesii*; Amp 1 and Amp 2 for *N. ampullaria* and Kam 1 and Kam 2 of *N. kampotiana*. The primers were evaluated with each other *Nepenthes* species. Finally, species-specific SCAR markers were successfully developed for *N. gracilis*, *N. ampullaria* and *N. kampotiana*. Application of these markers is feasible for identification of *Nepenthes* species in Thailand.

Key words: Genotype identification, *Nepenthes*, RAPD, SCAR marker, species identification

INTRODUCTION

Nepenthes is the single genus of the family Nepenthaceae with approximate 85 species originating from parts of Southeast Asia, Madagascar Australia. The islands of Sumatra and Borneo contain the largest number of endemic species (Clarke, 1997). *Nepenthes* species are dioecious carnivorous plants, with inconspicuous flowers lacking petals. The pitcher forms from a swelling at the tip of the leaf mid-vein. It functions by first, attracting the insects with nectar secretions and coloration and then, killing and digesting the insects. The breakdown products are absorbed to augment the plants nutrients uptake from the soil (Moran, 1996). *Nepenthes* species usually produce two morphological different pitchers. Young plants with a rosette stadium have lower or ground pitchers with mouth opening towards the tendril and wings situated along the pitcher wall. When the plant begins to climb, upper or aerial pitchers are produced. These lack the wings and the tendril forms on the backside of the pitcher. These two pitchers contrast so greatly, that a single species may be easily misunidentified as two different plants or species (Shivas, 1984).

Many Floras of *Nepenthes* have been published, for instances, Ridley (1967), Handerson (1974), Shivas (1984) and Clark (2002), but none originate from Thailand. Only Smittinand (1980) noted the existence of *N. kampotiana* Lecomte, *N. mirabilis* Druce, *N. smilesii* Hemsl. and *N. thorelii* Smittinand (2001) reported the species in Thailand, *N. ampullaria* Jack, *N. gracilis* Korth, *N. mirabilis*, *N. smilesii* and *N. thorelii*. Due to their interesting characteristics as carnivorous plants with attractive pitchers, these plants have high economic importance as ornamentals. Wherever it grows, *Nepenthes* rarely fails to excite the interest and curiosity of people.

On account of their fascinating beauty, wild *Nepenthes* species are often collected from the forest and sold in the market. Collectors may further breed hybrids to produce a diversity of pitcher characters. Natural hybrids can be possible. However, hybrid offspring rarely succeeds to develop into a wild population (Clarke, 2002). As a result, it has become difficult to find *Nepenthes* species growing in the wild.

A number of Polymerase Chain Reaction (PCR) based DNA markers, including Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR) and Amplified Fragment

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Length Polymorphisms (AFLP) techniques, have been used widely to investigate population genetic (Chaveerach *et al*., 2006). The RAPD markers are identified by using arbitrary primers and allow the quick construction of genetic maps for any plant species or the saturation of specific genomic regions with molecular markers. The short random primers used in RAPD analysis usually anneal with multiple sites in different regions of the genome and thus may amplify several genetic loci. In addition, the RAPD technique is sensitive to reaction conditions, which results in poor reproducibility. To overcome the problems associated with RAPD markers and to improve their utility in marker-associated selection, longer primers have been developed from RAPD fragments (Paran and Michelmore, 1993). These longer primers generate a Sequence-Characterized Amplified Region (SCAR), which can be particularly useful to follow the inheritance of the marked region of the genome. The SCAR markers are preferred over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions and they can potentially be converted into allele-specific markers (Ardiel *et al*., 2002).

The SCAR marker, a species-specific tool that can potentially be applied for molecular identification (He *et al*., 2006), has been developed for many plants including barley (Ardiel *et al*., 2002), bentgrass (Scheef *et al*., 2003), bamboo (Das *et al*., 2005), Indian gooseberry (Dnyaneshwar *et al*., 2006) and *Sinoclycanthus chinensis* (Ye *et al*., 2006), for example. It is feasible for users, than other methods such as DNA barcoding, in species identification.

The researchers have studied genetic diversity using molecular data (Chaveerach *et al*., 2007), species identification and sex determination using DNA fingerprinting (Pavlovic *et al*., 2007) and protein markers (Pinthong *et al*., 2009) of the genus *Nepenthes* in Thailand. We found out difficulty of identification of this high diverse plant group. Though species identification tools have been purposed but with limitation of application. A widely applicable tool is not yet established. This research aims to develop species-specific SCAR markers for identification of all *Nepenthes* species in Thailand.

MATERIALS AND METHODS

Plant materials: Eighteen individuals of *Nepenthes* species were collected, including 4 individuals each of *N. gracilis* Korth., *N. mirabilis* Druce, *N. smilesii* Hemsl. and *N. ampullaria* Jack and 2 individuals of *N. kampotiana* Lecomte. Young leaves were used for DNA extraction.

DNA extraction: Genomic DNA was extracted from the frozen leaves using the Plant Genomic DNA Extraction kit (RBC Bioscience). Quantity and quality of the DNA samples were examined by 0.8% agarose gel electrophoresis in TAE buffer strained staining with ethidium bromide. Then, DNA samples were diluted to a final concentration of 20 ng μL^{-1} in TE and these dilutions were used as templates for RAPD analysis.

RAPD analysis: Forty RAPD primers were screened on the two selected individuals of each species. Amplifications were carried out in 25 µL reactions containing 12.5 µL Go Taq® Green Master Mix (Promega), 0.5 µM primers and 20 ng of DNA template. The PCR condition is as followed: initial denaturation at 94°C for 3 min and following with 35 thermal cycles of denaturation for 1 min at 94°C, annealing for 1 min at 40°C, extension for 2 min at 72°C and final extension for 10 min at 72°C using a Gene Amp PCR System 9700 (Applied Biosystems). Amplification products were detected by 1.2% agarose gel electrophoresis in TAE buffer straining with ethidium bromide.

Cloning and sequencing of RAPD fragments: The selected bands were excised from the gel and purified with HiYield™ Gel/PCR DNA Extraction Kit (RBC Bioscience). The purified fragments were ligated into pCR®8/GW/TOPO® vector (Invitrogen) according to the manufacturer's instructions. The recombinant plasmids were transformed into Mach1™-T1^{R} (Invitrogen) competent cells and plated on selective media containing spectinomycin and X-gal. White colonies were picked from the plate and cultured for 4 h. The plasmid DNA was extracted using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). The identity clone products were confirmed by PCR and *Eco*RI digestion. The clones were sequenced at the Genome Institute, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.

Primer design and SCAR marker analysis: The SCAR primers consisting of 19-20 bases were designed from the sequence of the cloned species-specific DNA fragments using Primer 3 tool (http://biotools.umassmed.edu). The primers were tested for species specificity using the same amplification reactions as described above mixture with the same individuals as used in RAPD analysis and another two individuals of each *Nepenthes* species, excluding *N. kampotiana*.

RESULTS AND DISCUSSION

Identification of species-specific RAPD marker for *Nepenthes* species: Of the forty RAPD primers screened on selected ten accessions of five species, three RAPD

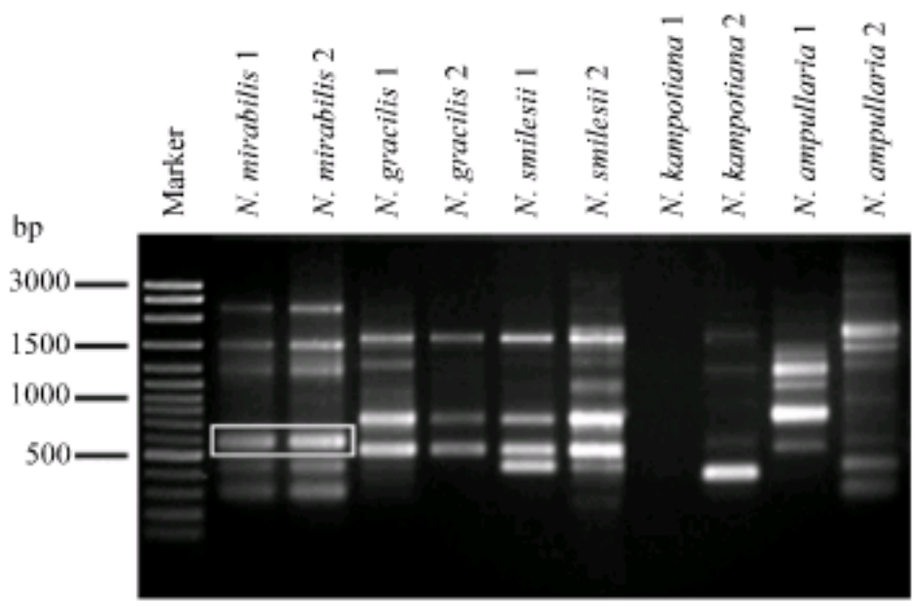

Fig. 1: RAPD patterns of *Nepenthes* species generated by primer 5'-GTACGCCCGA-3' showing specific band at approximately 600 bp (boxed) in *N. mirabilis*

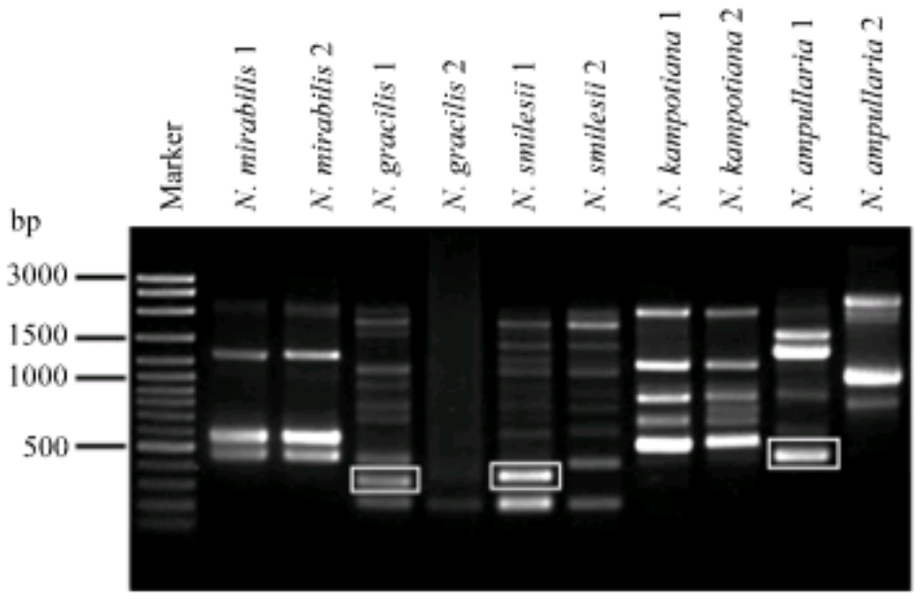

Fig. 2: RAPD patterns of *Nepenthes* species generated by primer 5'-CACAGGCGGA-3' showing specific bands at approximately 300, 400 and 500 bp (boxed) in *N. gracilis, N. smilesii* and *N. ampullaria*, respectively

Table 1: The three successful RAPD primers produced a specific band marker for each *Nepenthes* species and GenBank accession number for each sequence

RAPD primer (5'-3')	Plant species	Sequence size (bp)	GenBank accession No.
GTACGCCCGA	*N. mirabilis*	602	GQ166959
CACAGGCGGA	*N. gracilis*	379	GQ166960
	N. smilesii	420	GQ166961
	N. ampullaria	473	GQ166962
GAGCGTCGAA	*N. kampotiana*	1,017	GQ166963

primers (Table 1) produced distinguish banding patterns for each species. Amongst the amplified banding patterns, a specific band for each species is noted here. The first primer, 5'-GTACGCCCGA-3' (Fig. 1), gave a specific band of approximately 600 bp for *N. mirabilis*. The second primer, 5'-CACAGGCGGA-3' (Fig. 2), gave three specific bands of approximately 300, 400 and 500 bp for *N. gracilis*, *N. smilesii* and *N. ampullaria*, respectively. The third primer, 5'-GAGCGTCGAA-3' (Fig. 3), gave

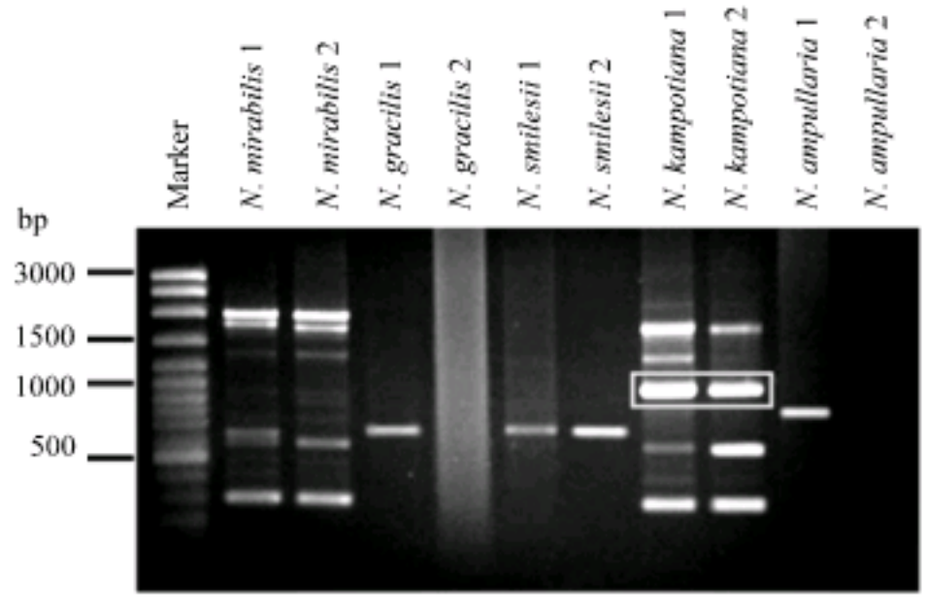

Fig. 3: RAPD patterns of *Nepenthes* species generated by primer 5'-GAGCGTCGAA-3' showing specific band at approximately 1,000 bp in *N. kampotiana*

a specific band of approximately 1,000 bp for *N. kampotiana*. These species-specific bands are candidate SCAR markers, thus were cloned and sequenced. Lengths of the DNA sequences are 602, 379, 420, 473 and 1,017 bp for *N. mirabilis* (Fig. 4), *N. gracilis* (Fig. 5), *N. smilesii* (Fig. 6), *N. ampullaria* (Fig. 7) and *N. kampotiana* (Fig. 8), respectively. First and last ten nucleotides of the sequences match completely with the corresponding RAPD primer used for each of the bands.

SCAR markers developed from species-specific RAPD markers: A pair of 19-20 bp primers for each species was designed from sequences of the species-specific RAPD band sequences. The designed sites and the lengths of expected amplified products are shown on Fig. 4-8 and Table 2.

Primers Mir 1/Mir 2 and Mir 1/Mir 3 did not produce species-specific bands for *N. mirabilis* as also produced bands for *N. smilesii*, *N. ampullaria* and *N. kampotiana* as shown in Fig. 9A and B. As same appearing in *N. smilesii*, there are no species-specific bands from primers Smi 1/Smi 2 and Smi 1/Smi 3. These primers also produced bands in *N. mirabilis*, *N. gracilis* and *N. kampotiana* as shown in Fig. 9C. Bright and easily identified bands being SCAR markers were found for *N. gracilis*, *N. ampullaria* and *N. kampotiana* as shown in Fig. 9D-F, respectively. Therefore, the SCAR primer pairs designed are proved to produce species-specific markers of these three studied species.

Due to their fascinating beauty, interesting characteristics as carnivorous plants with attractive pitchers, *Nepenthes* species have high economic importance as ornamentals, wild *Nepenthes* species are often collected from the forest and sold in the market and as a result, it may become difficult to find *Nepenthes* species growing in the wild. Therefore, there is high

GTACGCCCGAGAT**CCTTGGAGGAGACCGTTGTA**GCCCATGCCTTATGGC
Mir1
ATGTCTTCGTTACTAACGAGAGTGGCTTGGCCATTCCGCCGTCGTTGTG
TGCTTCCTTCCGGGCTGTGGCCCAGCCGGGTTGAGCGGATTCTTTGACT
ACCCCCGCGGCTGCCTTCACCTGTTAGGCGACGGCTTTCGCAGGTGTCT
ACTCCGCGAGCCATGCCAGGGCGCTAACCGCGGAAGGAGTGCGAGGTCA
GTAGCCCTCTGTGGAAGTTGGGCTTGACACCGAACTTTCCCGGAGTGCC
TGGCTTCAGTGCTTCCTGGACCCCGGTGGATT**GAATGCTTGAGACTCCC**
Mir3
ACCGGGGTACGCTCCCTGCTTCCCGCTCAGTTCACCCGGTGAAATACCG
ACTGCTTCCACCGTCGTCCGCCGGGCTCTCCTGGGACGAGGTACAGGAG
CATACCGGGCTTCGCACTACAGTCTTCTTTCGTCTTTCTCGCAGTTTCA
CGGTTGTAGATCTTATACTTTGTAAGAAAGTATTGATTAATAAAAGAAG
TAGCCTCTTTTGTCTTCAATTTT**CGTCTTGCCTTCGTAAATGC**TTTTCT
Mir2
GTTG**TCGGGCGTAC**

Fig. 4: Nucleotide sequence of *N. mirabilis* (GenBank accession No. GQ166959) from RAPD primer 5'-GTACGCCCGA-3' showing sites of SCAR primers

CACAGGCGGATGCGGTGTGAAGGCTGAGCCGGCACGTGAAGGCGGGACA
GGAACATAGATGACCATGTGCACCGCAGATTGTGGAAAAGATTGTATTA
GAATCGAGGACCATCTCGTTAAAGAACGAATAATGACACTTCCCAATGA
Gra1
CATGATATCTGTCGTTCAATATCAGCTAACCTTCCCCACTGACCGGTAA
ATATAGAGCAAATACTCATGTGGCTACGGGTACAAGTGCTTCATTGGTG
GCGACGCTGCCCATTCTCATGGCGGTCGGGCTGC**CTGCAACTTGGGAGT**
Gra2
CGATTCCAGAGTCGTGGCCGACCGTGTCGTCCCTGTAGAGTTTTTTGCG
CAATCTCATACCCCATATTCTCCTGC**TCCGCCTGTG**

Fig. 5: Nucleotide sequences of *N. gracilis* (GenBank accession No. GQ166960) from RAPD primer 5'-CACAGGCGGA-3' showing sites of SCAR primers

CACAGGCGGAGGAGCAAAAC**CTCAAGGAGAGGCTCTTTCG**GGTGATCTC
Smi1
GCGTGCTCCCCGAGCGTCGCCCCTTCTCATGTATGGTTGCTTCAATCTG
GCCTTCCTCTACTGTTGCCTGCCTTGGGAGTGCCTTTGATGTTGTTTCT
TATTCAGGCTCTAGGTAGTGCTAACCCTTTTGCTGTGTTGTCTACCCTC
CTCCACGGGAACTTGTAGCAGGTGATCTCCAGCGGAAGAGGCTAATTCG
CATCTCTCGAAGAGGAGAATGGGCGCCTGCAGCAGAGAGTGGCGCAG**CT**
GGAAGCCCTACTCGTCGCCTCGGAGGACCATATTGCTAGGTTGATTAGA
Smi3
GGCATGGTTGGACGGCTTTCTCCATCGAAATCGGAGCTGGCGTTGGA**GA**
CTCGGTATTGGGACGGTA**TCCGCCTGTG**
Smi2

Fig. 6: Nucleotide sequence of *N. smilesii* from RAPD primer 5'-CACAGGCGGA-3' showing sites of SCAR primers

CACAGGCGGAGGAATTAAAACGAAGGGCTGGTCTGTGAGGAAATGTCAA
AGTAAAGCAAAGAAGAGAGACAGAGAAGTCTTGGAGGAGAAAAATAGGA
CAGGACACAGGAGAGAGGAGTGAGGAGTGAAAGTA**GAAAACGCAAGGAG**
Amp1
AGGTTGGCGTTCCCCTAAATACGCACAGGCAGCGAAGAAAGCACAAAAG
TAAGAAGAATTAAATATTTGATTGCTTCCGAGTTTTTCTAAAGAAAGAA
AGCAGTCACTCTTCCTCCCAGCTGCCTAATATCCGCCTTCTTTTCCTTT
TTTTGTCCCTCCCCCACCTGTTCTCCTCTTGGGGCCGCGTGTGGAGGCC
CTAGCTGATATATGCTTGGTGGTGTATTTGACAAGAGCACAATAATAGG
GTCTTCAAGAATACCTCTTCCCCGATGC**GTGCAGTGGTTCATCTCGTG**C
Amp2
AAGGGGACATCCCATCTCGCCA**TCCGCCTGTG**

Fig. 7: Nucleotide sequence of *N. ampullaria* from RAPD primer 5'-CACAGGCGGA-3' showing sites of SCAR primers

GAGCGTCGAACGAATACCACGAACACTAGGCGCCATGGAGGAACAGGGA
AATTGAAAAGGGGGAAGGAGTAAAAGAGAGAAGCCGTCAACGAATACCA
TGAATGCCGCACCGTCAAGGGCGGCCCACTTGGGTGAAGTAGCCCAAGC
CCAGTTCACCGACCCAGCCCGCCAGCCTGTGGGTTGGTGGGGAGATCCA
CCCGTGGCATTTCCTTGATTGCTTG**CGAACGCCACACAACTTAGA**AGGG
Kam1
ATTCTTACCAAAATTGCCTTGATTTCGGTAAATATCCTACACGTATTTC
TAGATTTACTCGCCTCCCGAAATGGCAAGCGATAGCTGGCCAAATCCTA
TGTAGAATCATTTGCCGCCCAAGGAAAGAAACTCTAACTCAATGAATCG
TACCCCTGAAGGATGCTATCGTAAGGTAATCTCCACGACCCTCTTGAAG
AAGGGAACGATATCGAAGGGCAGTAGAACTTGCCTTGAAAGGCGCTTGC
AATCATCACACCAGTTCAGCAATCACTAACCATTGAAAACAGCAGAGAC
TCGACTGCCTTCATCAGCTATAAATACCCCTTGCGACAGACGGTTAAAG
GGACATGAAAAAATAAACAGATCCCTCCTTCCAGTGTCGATAGGCCACC
CGCGATGCAAACCGCTGAGTCACAAAAAGAATACATTGAGAGGAGCCTG
CAACATATTGCAGAAAAAGTAGATAATCTCTCACCATCTGAAGACGTTC
CACGTTGAAGCCCATCCTCATTCAAGCTGCCATCTAGGCATGCCCTCCA
CGCAAGCTGTCGTAACTCAGGCCCATCCTCCGTGTCAAACTCAACCCCA
CTTCTGTGTATTATTCTCGCATTTGCTCATCTTTCTATTTTTCGCAAAA
GATTATTAATTTGAGTATCGGAGGATAAATCGGGGAGCGAGTCCCGTGA
TTTTTGTTTGACATCTTGTCTTCAATTGTGGTGCGTAGGATCCAAGTGC
GAAGCTACTAATCC**AGACCTGTTTCGATTCGACGCTC**
Kam2

Fig. 8: Nucleotide sequence of *N. kampotiana* from RAPD primer 5'-GAGCGTCGAA-3' showing sites of SCAR primers

Table 2: Details of SCAR primers designed from RAPD marker sequences of *Nepenthes* species

Plant species	SCAR primers	Type	Sequence (5'-3')	Expected product fragment size (bp)
N. mirabilis	Mir1	Forward	CCTTGGAGGAGACCGTTGTA	Mir1/2, 569
	Mir2	Reverse	GCATTTACGAAGGCAAGACG	Mir1/3, 333
	Mir3	Reverse	GGTGGGAGTCTCAAGCATTC	
N. gracilis	Gra1	Forward	GAATCGAGGACCATCTCGTT	Gra1/2, 200
	Gra2	Reverse	AATCGACTCCCAAGTTGCAG	
N. smilesii	Smi1	Forward	CTCAAGGAGAGGCTCTTTCG	Smi1/2, 390
	Smi2	Reverse	TACCGTCCCAATACCGAGTC	Smi1/3, 291
	Smi3	Reverse	CGACGAGTAGGGCTTCCAG	
N. ampullaria	Amp1	Forward	GAAAACGCAAGGAGAGGTTG	Amp1/2, 307
	Amp2	Reverse	CACGAGATGAACCACTGCAC	
N. kampotiana	Kam1	Forward	CGAACGCCACACAACTTAGA	Kam1/2, 792
	Kam2	Reverse	CGTCGAATCGAAACAGGTCT	

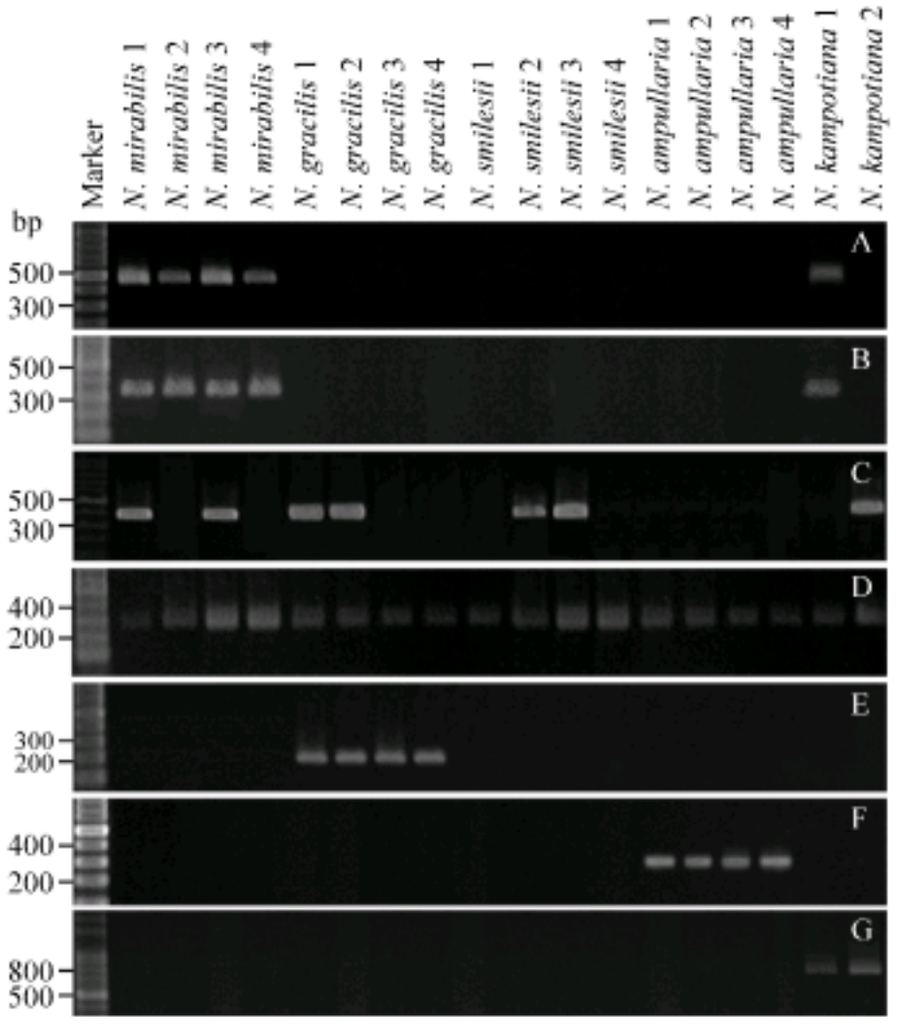

Fig. 9: Specific bands from primers (A) Mir 1/Mir 2, (B) Mir 1/Mir 3, (C) Smi 1/Smi 2, (D) Smi 1/Smi 3, (E) SCAR markers of *N. gracilis* from primer Gra 1/Gra 2, (F) *N. ampullaria* from primer Amp 1/Amp 2 and (G) *N. kampotiana* from primer Kam 1/Kam 2

attempt to grow the plants for commercial. However, it is difficult to select a right species for a right objective especially for immature plant. Moreover, they are dioecious plants leading more difficulty for identification.

Species identification, species diversity and genetic diversity of the genus *Nepenthes* in Thailand have long been observed by the authors. The first research evaluated, by Inter-Simple Sequence Repeat (ISSR) analysis, that *N. mirabilis* showed high genetic diversity (Chaveerach *et al*., 2006). Consequently, by ISSR and RAPD analyses, species identification and sex determination were accessible (Povlovic *et al*., 2007). However, identifications by means of DNA fingerprint have limitation of application. Species identification using protein marker has been also investigated (Pinthong *et al*., 2009) with limitation of application.

Sequence Characterized Amplified Region (SCAR) marker has been proven to be an efficient and available tool for quick and accurate species identification. From present result studied. The SCAR markers are successfully developed in *N. ampullaria*, *N. gracilis* and *N. kampotiana*. Of *N. gracilis*, SCAR band was generated in only an individual which was firstly cloned, sequenced and SCAR primer designed. Possibly, DNA templates of the other individuals were broken or nucleotide variation at priming sites, so, there has appeared no band. For *N. mirabilis* and *N. smilesii*, SCAR primers can also produce bands in the other species. Therefore, there have no SCAR markers in these two species.

Although, the SCAR marker is very useful application for authentication of the *Nepenthes* or other plants, nucleotide variation or mutation may be occurred at priming sites when the long time is over especially, affecting from environmental changing. With these reasons the developed SCAR marker may be applicable for a time period.

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