

A New Classification of Thailand's *Nepenthes* Species by Genetic Analysis of AFLP Markers

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Abstract

Nepenthes species are well known for their attractive, ornamental insect-catching pitchers. Characteristics based upon the vegetative parts of these plants are of limited use for taxonomic purposes. This study analyzed genetic relatedness of 13 *Nepenthes* species dispersed throughout Thailand using the Amplified Fragment Length Polymorphism (AFLP) marker system. The results of DNA extracted from leaves of *Nepenthes* species using modified Doyle and Doyle (1990) revealed a new method for genomic DNA isolation of both the highest quality and highest quantities of *Nepenthes* to date. All of the 12 primers screened produced highly reproducible AFLP bands with 100% polymorphism. The number of AFLP fragments generated per primer ranged from 103 to 153 with fragment sizes varying from 100 to 500 bp. A total of 1,461 discernible DNA fragments were detected, of which 73.79% were polymorphic and 26.21% were monomorphic. *Nepenthes* samples formed a tight cluster in six groups. The dendrogram constructed from AFLP analysis successfully separated the *Nepenthes* samples individually by geographical area and species. *Nepenthes* species collected from related ecological habitats appeared in the same groups and differed from the others. The results of this study clearly explain the relationship of *Nepenthes* species growing in Thailand.

INTRODUCTION

Nepenthes are popularly known as tropical pitcher plants or monkey cups. This genus comprises roughly 140 species which are scattered throughout the Old World tropics, ranging from South China, Indonesia, Malaysia, Philippines, Madagascar, Australia, India and Sri Lanka. However, the greatest diversity occurs on Borneo and Sumatra, with many endemic species. (Meimberg and Heubl, 2006). Normally, these plants are characterized by their insect-catching pitchers. Delimitation of species is usually difficult as the pitchers exhibit high variation within species and populations. Consequently, characteristics based upon the vegetative parts of these plants are of limited use for taxonomy (Cheek and Jebb, 2001). According to the survey of *Nepenthes* species in Thailand, 15 species were found (Figs. 1 and 2). The constraints of vegetative taxonomical classification occurred among these varieties. Recently, various molecular approaches have been devised to overcome these constraints (Meimberg and Heubl, 2006; Mookamul et al., 2007). Here we report on the successful classification of the *Nepenthes* species in Thailand by using Amplified Fragment Length Polymorphisms (AFLP), as well as a rapid, effective and reproducible isolation of genomic DNA extracted from leaf of *Nepenthes* sp.

MATERIALS AND METHODS

Thirty plant samples of mature *Nepenthes* species throughout Thailand were used in this study. We were unable to collect *N. sanguinea* and *N. thai* that grow in Prutaodang swamp forest, Narathiwat province, due to civil unrest in this collecting area. All of the plant samples were kindly supplied by Khun Trongtham Kruetreepradit, Thailand *Nepenthes*' survey from Nakhon-sawan province. Four different DNA extraction methods, based upon Saghai-Marooof et al. (1984), modified Doyle and Doyle (1990), Madyod et al. (2010) and Wuthisuthimethavee et al. (not published) were used to extract samples from

young leaves of these plants. AFLP analysis was conducted followed by Vos et al. (1995) with 12 primer combinations. Banding from the agarose gel were scored as present (1) and absent (0) and used as a raw data matrix. The dendrogram was then generated by UPGMA with the DNA fingerprinting II program version 3.0 (Bio Rad).

RESULTS AND DISCUSSION

DNA extracted from leaves of *Nepenthes* species using modified Doyle and Doyle (1990) produced the highest quality samples of up to 20 ng μL^{-1} with no DNA degradation. In *Nepenthes mira*, Fleischman and Heubl (2009) reported that DNA extracted from leaf using both modified and standard protocol of the NucleoSpin® Plant Kit (Macherey-Nagel, 2007) produced both the highest quantity and quality of genomic DNA (12.0 and 18.9 ng μL^{-1} , respectively). They also stated that leaf tissue of carnivorous plant taxa contains high contents of polysaccharides, phenolic compounds and other secondary plant metabolites that interfere with DNA isolation and amplification. On the contrary, resultant genomic DNA extracted with modified Doyle and Doyle (1990) in this research showed no DNA degradation or smear. All samples were successfully used for PCR analysis. Thus, different methods of DNA extraction can have major influence on both quality and quantity of DNA in *Nepenthes* sp.

The results from AFLP technique showed that all 12 primers screened produced highly reproducible AFLP bands and displayed 100% polymorphism. The number of AFLP fragments generated per primer ranged from 103 to 153 with fragment sizes varying from 100 to 500 bp. A total of 1,461 discernible DNA fragments were detected, of which 73.79% were polymorphic and 26.21% were monomorphic (Table 1 and Fig. 3). In pomegranate, Jbir et al. (2008) and Yuan et al. (2007), observed a polymorphism of 57.5% and 73%, respectively by using the AFLP technique. These results probably occurred due to the large number of polymorphic loci revealed by AFLP increasing the ability to examine inter-specific genetic differences of these clones.

Nepenthes samples formed tight clusters in six groups and the dendrogram constructed from AFLP analysis successfully separated the *Nepenthes* sampled individuals by geographical area and species. *Nepenthes* species collected from related ecological habits appeared in the same group and differed markedly from the other groups (Fig. 4). Results from AFLP banding clearly confirmed the relationship of *N. mirabilis* group members. All of these species *N. mirabilis* ‘Nakhon Si Thammarat’ and ‘Khura Buri’ and *N. mirabilis* var. *globosa* or ‘Viking Phang-nga’ and ‘Trang’ are in the same group and separated from *N. andamana* or ‘Tiger Phang-Nga’. The other species of the “Tiger” group from the South namely, *N. kongkandana*, *N. suratensis* and *N. kerrii* were identified as being from the same related group. Whereas, the species of the “Tiger” group from the North-East of Thailand namely, *N. chang*, *N. kampaotiana*, *N. smilesii* are separated in the other groups. Plants from these groups are clearly identified as species not sub-species as opposed to previous groupings based upon morphological data. The last two groups, namely, *N. gracilis* and *N. ampullaria* were classified in different groups based upon their genetic code, geographic location and morphological appearance. From our results, it can be concluded that AFLP technique can successfully be used to determine the identification of *Nepenthes* species in Thailand. This technique requires few expenses, a short time and simplified methodology.

CONCLUSIONS

This is the first reported instance of a rapid, effective and reproducible isolation resulting in both high quality and quantity genomic DNA extraction from leaf of *Nepenthes* sp. AFLP analysis using 12 primers screening which has successfully identified *Nepenthes* sp. in Thailand, thus proving that information at molecular level is of a great value when used for plant species determination.

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Tables

Table 1. AFLP primer combinations of analyzed *Nepenthes* species in Thailand.

Primers combination	TNB	NMB	%MB	NPB	%PB
ER-AT/MS-CGA	110	15	13.64	95	86.36
ER-AT/MS-CAT	110	20	18.18	90	81.82
ER-AT/MS-CTA	120	18	15.00	102	85.00
ER-AT/MS-CCA	132	24	18.18	108	81.82
ER-AT/MS-CAG	150	19	12.67	131	87.33
ER-AT/MS-CGT	147	19	12.93	128	87.07
ER-AA/MS-CGA	129	11	8.53	118	91.47
ER-AA/MS-CAT	111	43	38.74	68	61.25
ER-AA/MS-CTA	110	34	30.91	76	69.09
ER-AA/MS-CCA	103	72	69.90	31	30.10
ER-AA/MS-CAG	116	53	45.96	63	54.31
ER-AA/MS-CGT	123	55	44.72	68	55.28
Total	1,461	383	329.36	1,078	870.90
Average	121.75	31.92	27.45	89.83	72.58

TNB = total number of bands; NMB = number of monomorphic bands, %MB = percentage of monomorphic bands, NPB = number of polymorphic bands and %PB = percentage of polymorphic bands.

Figures

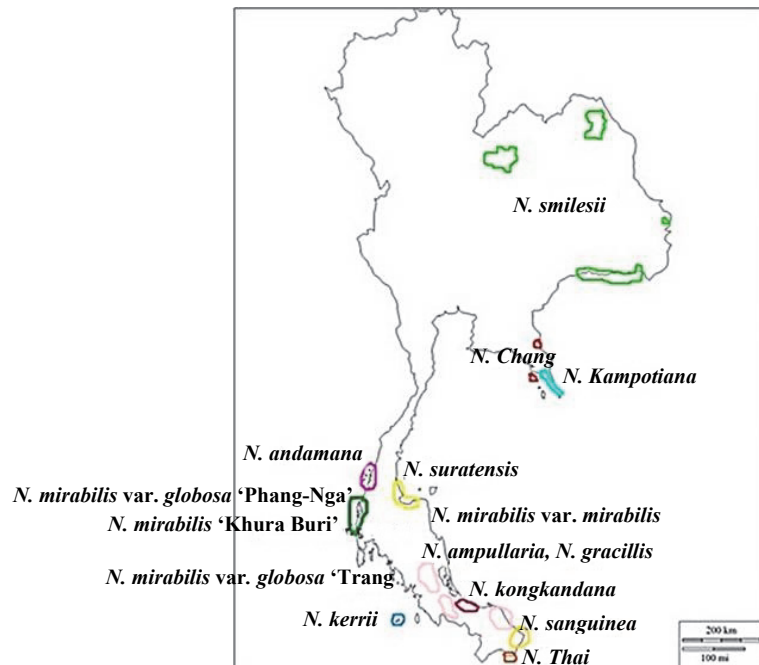


Fig. 1. Map of Thailand indicating *Nepenthes* sampling.
(modified from <http://api.ning.com>).

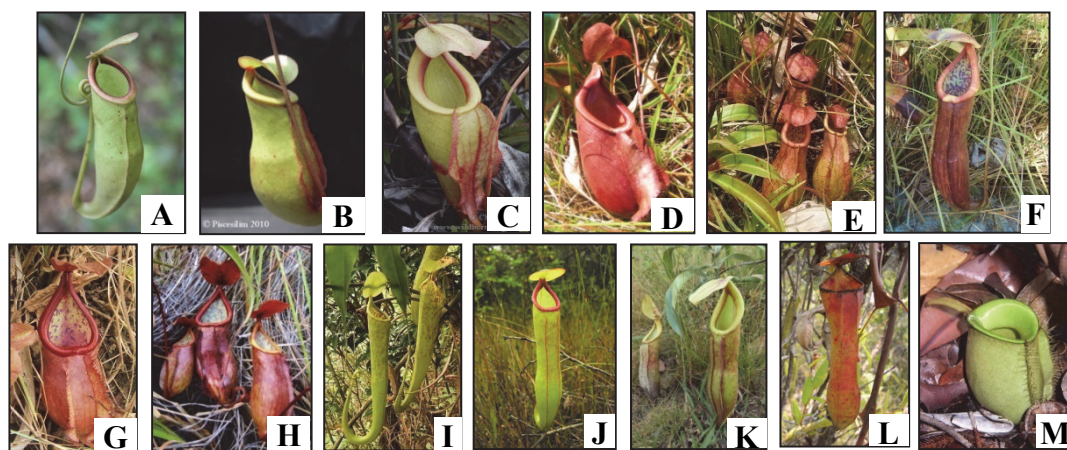


Fig. 2. *Nepenthes* species distributed in Thailand. A = *N. mirabilis* var. *mirabilis*, B = *N. mirabilis* 'Khura Buri', C = *N. mirabilis* var. *globosa* 'Phang-Nga', D = *N. mirabilis* var. *globosa* 'Trang' .E = *N. andamana*, F = *N. kongkandana*, G = *N. suratensis*, H = *N. kerrii*, I = *N. chang*, J = *N. kampotiana*, K = *N. smilesii*, L = *N. gracilis* and M = *N. ampullaria*. (Figs. A, E, F, G, H, I, J, K, L, M and N from en.wikipedia.org/wiki/Nepenthes, B from www.wildborneo.com.my, C from http://img269.imageshack.us/img269/2870/viking01_current.jpg and D from <http://www.carnivorousplants.it/mcp22.JPG>.

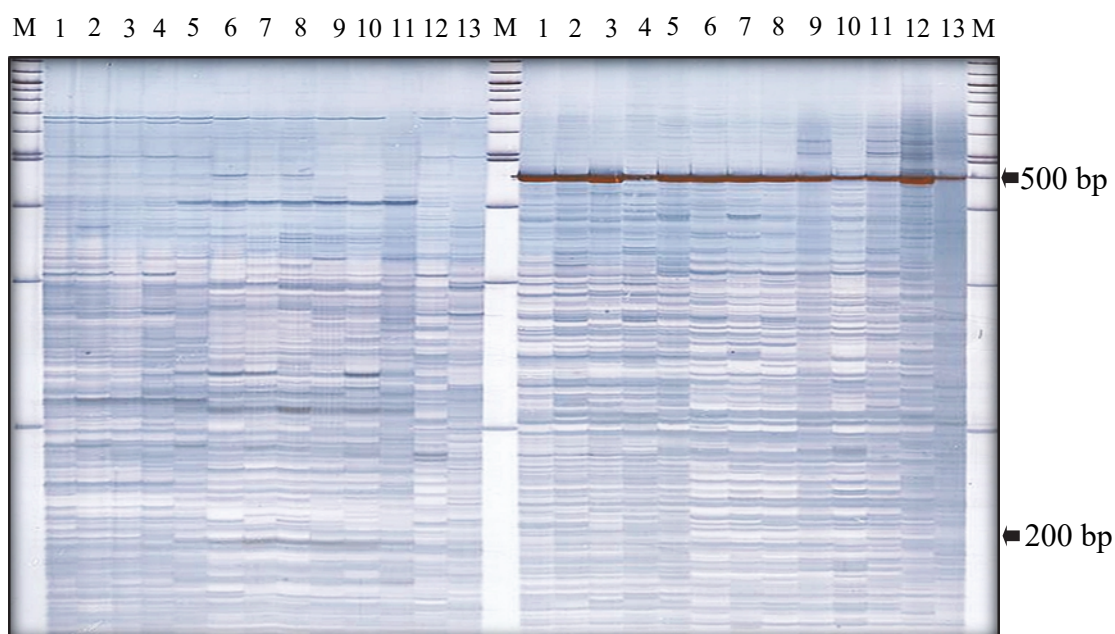


Fig. 3. A sample of the AFLP banding pattern from primer ER-AT/MS-CCA and ER-AT/MS-CAG. M = 1 kb molecular weight markers, 1-13 = *N. mirabilis* var. *mirabilis*, *N. mirabilis* 'Khura Buri', *N. mirabilis* var. *globosa* 'Trang', *N. mirabilis* var. *globosa* 'Phang-Nga', *N. andamana*, *N. kongkandana*, *N. suratensis*, *N. kerrii*, *N. chang*, *N. kampotiana*, *N. smilesii*, *N. gracilis* and *N. ampullaria*, respectively.

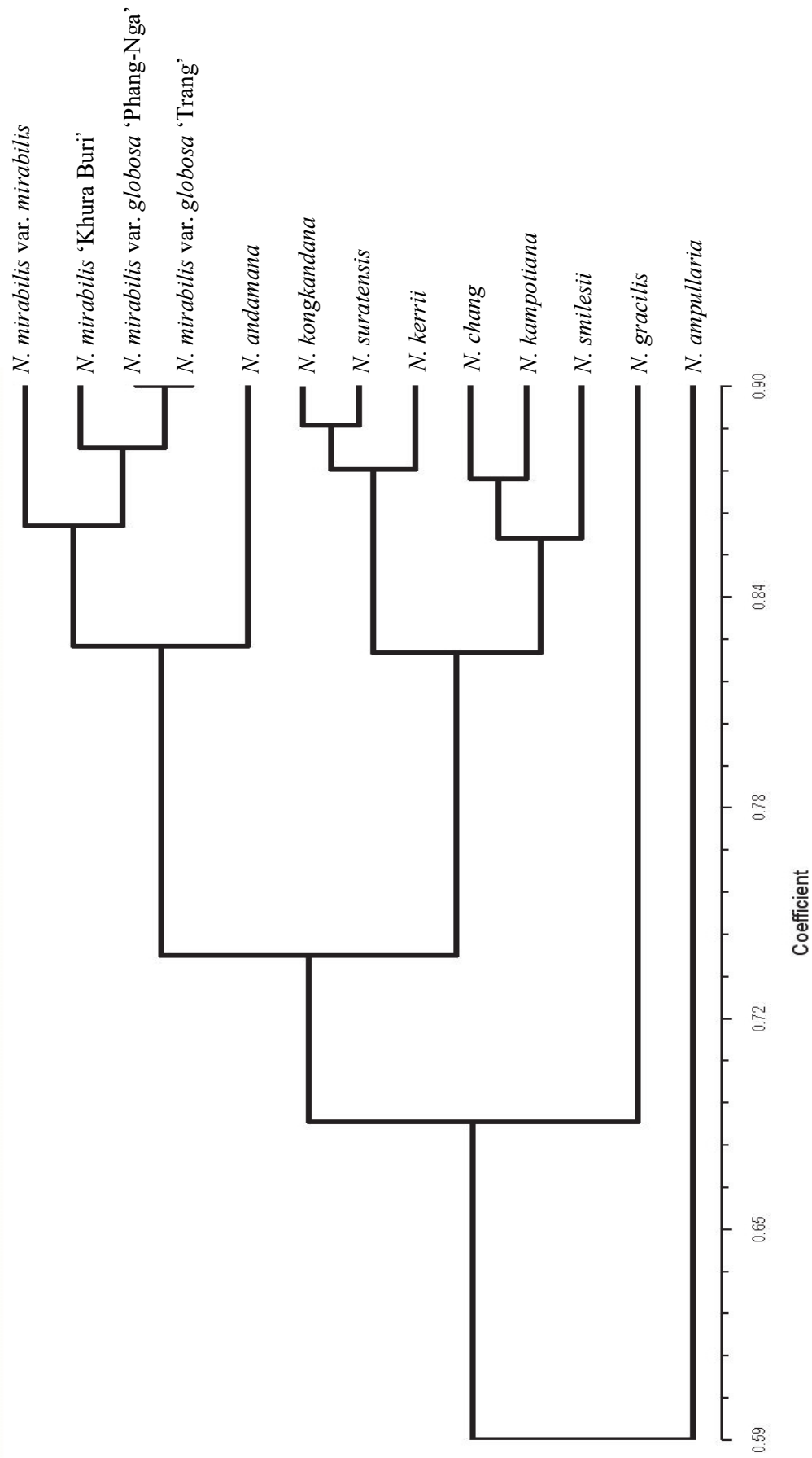


Fig. 4. Dendrogram depicting from 12 AFLP primer combinations produced by UPGMA analysis classifying the *Nepenthes* species in Thailand by their geographical area and species.