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Species Identification and Sex Determination of the Genus *Nepenthes* (Nepenthaceae)

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Abstract: *Nepenthes* species are well known for their ornamentally attractive pitchers. The species diversity was randomly surveyed in some conservation areas of Thailand and three species were found, namely *N. gracilis* Korth., *N. mirabilis* Druce. and *N. smilesii* Hemsl. Young plants as unknown species from Chatuchak market were added in plant sampled set. Thirty two Inter Simple Sequence Repeat (ISSR) primers were screened and 13 successful primers were used to produce DNA banding patterns for constructing a dendrogram. The dendrogram is potentially power tool to identify unknown species from Chatuchak market, differentiate species population, population by geographical areas and sex determination. The geographical area of *N. mirabilis* was specified to Southern and Northeastern regions and finally, subdivided into exact areas according to province. Male and female plants of *N. gracilis* at Phu Wua Wildlife Sanctuary and *N. mirabilis* at Bung Khonglong non-hunting area were determined. Two unknown species from Chatuchak market were analyzed to be *N. mirabilis* with the genetic similarities (S) 77.2 to 84.7. Be more sex specific in all sample studied, 37 Random Amplified Polymorphic DNA (RAPD) primers were investigated. The result shows that only one RAPD primer show high resolution results at about 750 bp specific male-related marker.

Key words: ISSR, *Nepenthes*, sex determination, species identification

INTRODUCTION

Nepenthaceae is represented by a single genus *Nepenthes* which is commonly known as the tropical pitcher plant. It consists of about 85 species (Clarke, 2002) originating from parts of Southeast Asia, Madagascar and Australia. The islands of Sumatra and Borneo contain the largest number of endemic species.

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 nutrient 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 misidentified as two different plants or species (Shivas, 1984).

Many Floras of *Nepenthes* have been published, for instance, Ridley (1967), Henderson (1974), Shivas (1984) and Clarke (2002), but none originate from Thailand. Only Smittinand (1980) notes the existence of *N. campotiana* Lec., *N. mirabilis* Druce., *N. smilesii* Hemsl. and *N. thorelii* Lec. in Thailand. Due to their interesting characteristic 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.

The genetic similarity (S) can be used to measure the relatedness of samples (Nybom and Hall, 1991; Welsh *et al*., 1991). The method of depicting the results of a phenetic analysis is by way of a branching diagram called a dendrogram produced by cluster analysis which is a method for grouping of Operational Taxonomic

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Units (OTUs). The result is a branching diagram that connects all of the OTUs and OTUs clusters at levels corresponding to their degree of similarity. By selecting an appropriate range of S to represent a given level in the taxonomic hierarchy, the taxonomists may then recognize species, genera, etc. Groups in which all OTUs, have similarities between 85 to 100% might be recognized as part of the same species, while a 65% criterion might be used for genera. However, the ultimate interpretation of the dendrogram is dependent upon the taxonomist's knowledge of the OTUs (Weier *et al*., 1982).

Traditionally, morphological characters have been used to characterize levels and patterns of diversity. Since these traits represent only a small portion of the plant genome and are influenced by environmental factors, they have limited utility for describing the potentially complex genetic structure which may exist within and between taxa (Avise, 1994). Various molecular approaches have been devised to overcome these constraints (Soltis and Soltis, 1990). 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 Length Polymorphisms (AFLP) techniques, have been used widely to investigate population genetic. ISSR markers have proven to be extremely variable and sensitive enough to differentiate cultivars and natural populations (Wolfe *et al*., 1998). These markers of genetic variation are generally independent of environmental factors and more numerous than phenotypic characters, thereby providing a clearer indication of the underlying variation in the genome.

Chaveerach *et al*. (2002) studied the genetic diversity among geographically separated populations of *N. mirabilis*. There is the great genetic diversity supporting the broad range of distribution sites of *N. mirabilis*, which would require high genetic diversity to adapt to survive in various environments that can be found between northeastern, central and southern Thailand. Mantel tests reveal that geographical distance is an important factor for affecting the genetic distances among populations.

Therefore, this research is the second steps of the genus before studying in depth of interesting genes which is performing in our laboratory. The objectives of this study were examined sex differences, identify unknown species of the young plants from markets or wild. ISSR and RAPD markers were used as OTUs for reaching these objectives.

MATERIALS AND METHODS

Plant materials: Collections were performed with exploring their growing areas during May to October 2005. Young leaves of two male and two female plants of *Nepenthes mirabilis* Druce., *N. gracilis* Korth. and *N. smilesii* Hemsl. including out group *Drosera indica* L. were collected from six locations including Chatuchak Market as shown in the Table 1 and Fig. 1. Leaf materials were immediately dried using silica gel then transported to the lab and stored at -70°C until DNA extraction.

Mature sampled specimens were identified by Hooker (1885), Ridley (1967), Henderson (1974), Shivas (1984), Moran (1996) and Clarke (2002).

This research was conducted since May 2005 at the Molecular Laboratory, Department of Biology, Faculty of Science, Khon Kaen University, Thailand.

DNA extraction: Genomic DNA was extracted from dried leaves using the QIAGEN DNeasy mini kit. The DNA was evaluated with 0.8% agarose gel electrophoresis stained with ethidium bromide and the quality and quantity of the DNA samples were determined by gel document instrument following the UPGMA with the DNA fingerprinting II program version 3.0 (Bio Rad). Then, the DNA samples were diluted to a final concentration of about 20 ng in TE and these dilutions were used as the DNA template in PCR reaction.

ISSR and RAPD analysis: Amplifications were carried out in 25 μL reactions consisting of reaction buffer, 2.5 mM of $MgCl_2$, 0.25 mM each of dNTPs, 0.65 μM of primers, 1.25 units of DNA polymerase (Invitrogen) and 5 ng DNA template. The 13 ISSR primers that successfully amplified clear bands are as follows: $(CA)_6GG$, $(CA)_6AC$, $(CA)_6AG$, $(CT)_8AC$, $(CT)_8TG$, $(GA)_6GG$, $(GA)_6CC$, $(GT)_6CC$, $(CAC)_3GC$, $(CTC)_3GC$, $(GACA)_4$, $CCCC(GT)_6$, $(GAG)_3GC$. The one RAPD primer that successfully amplified male-related marker band is TTCCGAACCC.

The reaction mixtures were incubated at 94°C for 3 min and the amplification was performed with the following thermal cycles: 35 cycles of denaturation for 1 min at 94°C, 2 min annealing temperature (Tm -5°C) for ISSR and 36°C for RAPD, 2 min at 72°C and 7 min final extension at 72°C. All amplification reactions were repeated at least two times using a PCR machine (Gene Amp PCR system 9700, Applied Biosystems). Amplified products were detected by agarose gel electrophoresis in TAE buffer (0.4 M Tris, 0.114% acetic acid, 1 mM EDTA, pH 8.0) and visualized by ethidium bromide staining.

Table 1: A summary of discovered plants, areas level of altitude and portions of Thailand

Sampled plants	Plant collection areas	Altitude (m)	Regions of Thailand
Nepenthes gracilis No. 12-13 females No. 14-15 males	Phu Wua Wildlife Sanctuary, Nong Khai province	300-400	Northeast
N. mirabilis No. 16-17 females No. 18-19 males	Bung Khonglong non-hunting area, Nong Khai province	200-300	Northeast
N. mirabilis No. 7-8 sex unknown	Ngao Waterfall National Park, Ranong province	100-200	South
N. mirabilis No. 9 female No. 10-11 males	Bangwan, Kuraburi district, Phang Nga province	100-200	South
N. smilesii No. 4 sex unknown	Phu Kradung National Park, Loei province	800-900	Northeast
N. smilesii No. 1, 2 and 3 sex unknown	Phu Khieo Wildlife Sanctuary, Chaiyaphum province	800-900	Northeast
Unknown species No. 5-6 sex unknown	Chatuchak market, Bangkok	Seller states that the plants were from Ranong province	

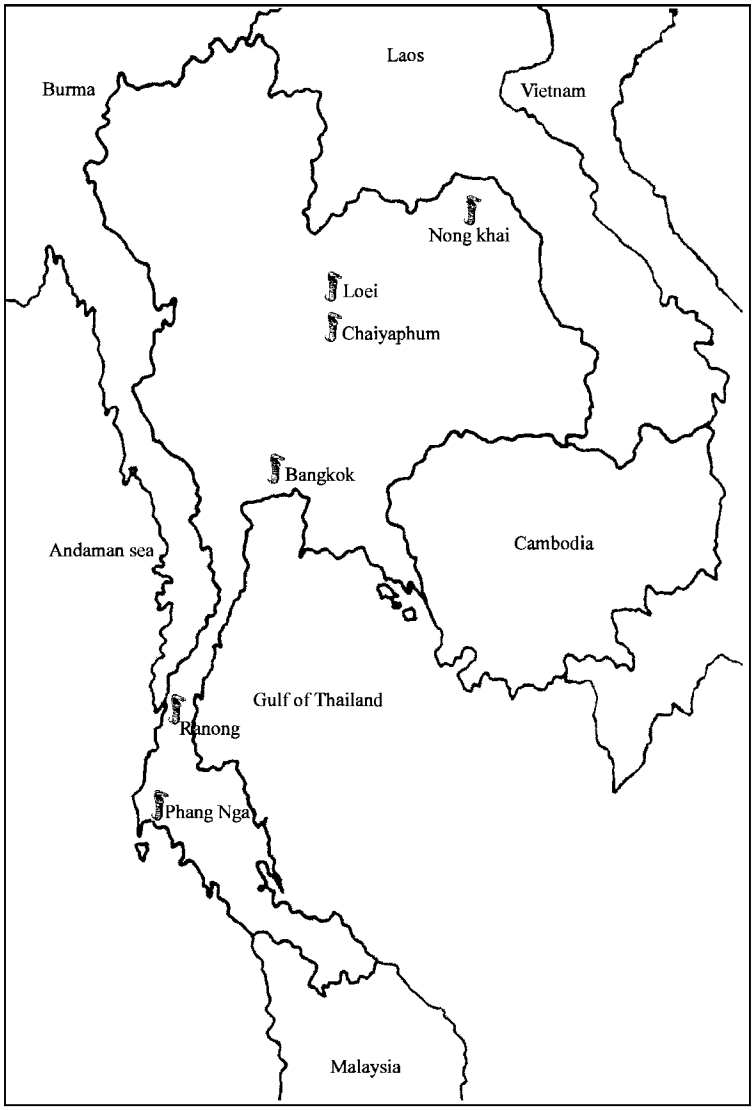

Fig. 1: Map of Thailand indicating sampling provinces of populations: *N. mirabilis* at Chatuchak market, Bangkok, Ranong, Phang Nga and Nong Khai.; *N. gracilis* at Nong Khai and *N. smilesii* at Loei and Chaiyaphum

Data analysis

ISSR data analysis: The total numbers of ISSR bands discerned from the agarose gel were documented as diallelic characters: present= 1, absent= 0; the ISSR are considered the dominant markers. The resulted bands were used to reconstruct a dendrogram following the UPGMA with the DNA fingerprinting II program version 3.0 (Bio Rad).

RAPD data analysis: The RAPD experiment was carried out in two stages. In the first stage, the DNA was pooled from all the male and female samples, separately and screening of primer was done on the pooled DNA. Thirty-five decamer primers were screened for differences in male and female samples. In this performing only one primer was identified which produced probable male-related band. In the next stage, the one produced probable male-related primer was used to confirm the presence and absence of bands in all the male and female entries, individually.

RESULTS

For species identification of young unknown samples from Chatuchak market, 32 ISSR primers were screened and 13 different polymorphism primers produced a total of 352 bands, ranging in sizes from 100 to 2500 bp (Fig. 2). ISSR analysis as shown in a constructed dendrogram successfully separated the sampled individuals by geographical area, species and sex (Fig. 3). The dendrogram revealed that geographical area was divided into two groups. The first group is *N. mirabilis* that includes samples from four subgeographical areas and young unknown species No. 5 and 6 from Chatuchak market. The male plants, No. 18 and 19 and female plants, No. 16 and 17, are separated. Memorable showing in dendrogram, the S values of young unknown species from Chatuchak market and *N. mirabilis* No. 7 and 8 from Ranong province are 77.2 to 84.7. These suggest that the species from Chatuchak market is *N. milabilis*. The second group is subdivided into two subgroups, from different geographical areas. The first is subdivided into two geographical areas, *N. smilesii* No. 1-3 from Phu Khieo Wildlife Sanctuary, Chaiyaphum province. The other one is *N. smilesii* No. 4 from Phu Kradung National Park, Loei province. The second subgroup is *N. gracilis* No. 12 and 13 both females and No. 14 and 15 both males, from Phu Wua Wildlife Sanctuary, Nong Khai province. The average S values of each species and individuals are shown in Table 2. The S values of individuals range from 70.33 of *N. mirabilis* to 88.22 of *N. gracilis*. The S values of each species pair range from 56.13 of *N. gracilis* and *N. mirabilis* to 66.29 of *N. gracilis* and *N. smilesii* indicating that they are in a genus.

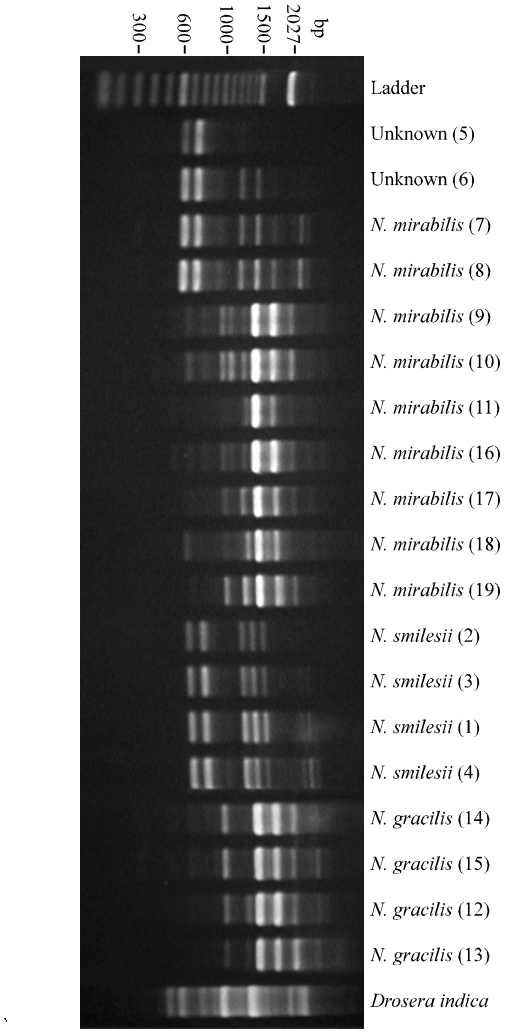

Fig. 2: A sample of ISSR banding pattern from primer $(CAC)_3GC$

Table 2: Average similarity index between three species of *Nepenthes*

Species	*N. mirabilis*	*N. smilesii*	*N. gracilis*	*Drosera indica*
N. mirabilis	70.33			
N. smilesii	59.59	85.90		
N. gracilis	56.13	66.29	88.22	
Drosera indica	42.27	47.80	51.82	100

For sex determination, 35 RAPD primers were screened and only one male-related marker primer produced a band, ranging in size about 750 bp appearing in both *N. mirabilis* and *N. gracilis*. The primer produces

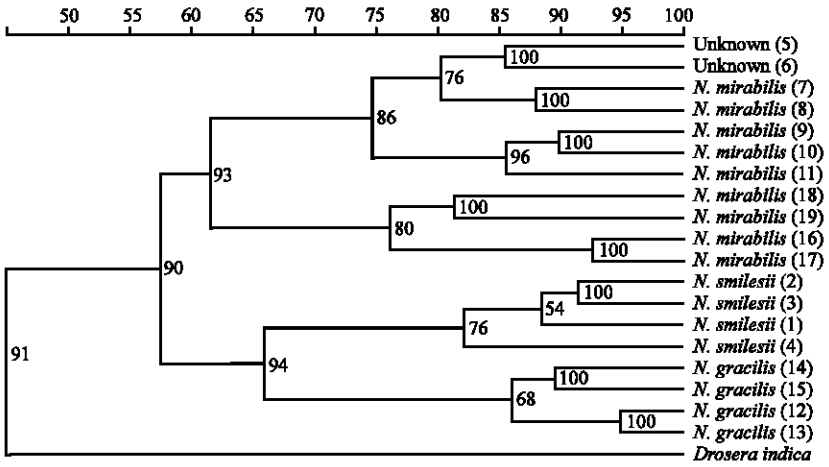

Fig. 3: Dendrogram depicting the thirteen ISSR primers produced by UPGMA analysis and used to classify the three population species, different geographical area collected and sex determination in some plants of the *Nepenthes* sp

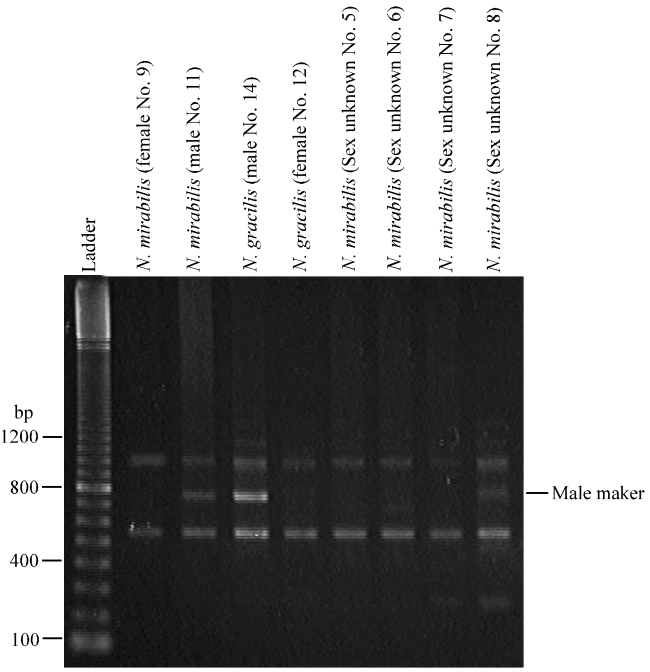

Fig. 4: RAPD patterns of male and female samples generated by primer TTCCGAACCC. Male-related marker is about 750 bp

identical banding patterns in the two species, 2 bands at about 550 and 1000 bp in female and 3 bands with additional male-related marker at about 750 bp (Fig. 4).

DISCUSSION

We sampled many different forest areas and in the cases where the same species were found in different areas, individuals were randomly selected for ISSR examination. We conclude that Northeastern Thailand is limited to three species of *Nepenthes*, specifically, *N. gracilis*, *N. mirabilis* and *N. smilesii* and of these three, only *N. mirabilis* can be found in South Thailand. However, we have not been to all regions of Thailand and it is likely that there are more species to be discovered in Thailand. For example, one of the authors has discovered

N. ampullaria at Plutudang swamp forest, Naratiwat province, South Thailand. Unfortunately, we were unable to collect this species to civil unrest in the collection area. We could not have higher number of samples or sites, because they are wild species which are always disturbed by humans picking to market. So, random sampling individuals were collected.

The S of each individual species is in a species range, at 70.33 to 88.22 indicating the same species, while the S between species is in a genus range at 56.13 to 66.29 showing the same genus (Weier *et al*., 1982).

The S of *N. mirabilis*, excluding the young unknown species No. 5 and 6, 70.33 is much lower than the other studied species. However, the authors decided that sampling individuals are still being a same species. Since for stable morphological characters, accordingly, the young unknown species No. 5 and 6 and *N. mirabilis* No. 7 and 8 in a branch of dendrogram show the S of 85.5 (Fig. 3), the No. 5 and 6 are *N. mirabilis* following to Weier *et al.* (1982). One additional proving, the No. 5 and 6, which is stated from the seller that the plants are from Ranong province, positions on a branch of *N. mirabilis* No. 7 and 8 collected from Ranong province.

The S of all individuals *N. mirabilis* indicate wide rang of 70.33 to 85.5, may be caused from they belong high genetic diversity, which agrees with its broad growing range (Chaveerach *et al*., 2006) and indicates that it is a hardy species that may be quite popular among plant hobbyists.

These primer sets for ISSR technique are highly reasonable results. The ISSR data that was used to construct dendrogram suggests the genetic similarity within and between species, differentiates the geographical area, separates sex in some species in a pair branch of dendrogram, distinguishes and identify species.

Gender is most often genetically determined in dioecious plants, either by sex chromosomes or by a genic system with expression of alleles at one or several loci on non-distinguishable chromosomes (Irish and Nelson, 1989; Durand and Durand, 1990). The presence of sex chromosomes in *Nepenthes* has not been reported. Formerly, we have observed that the chromosome number of Nepenthaceae are remarkably uniform 2n = 80 (data unpublished). RAPD banding patterns can serve as fingerprints for genotype identification in vegetatively propagated plant. Of many ways, sex determination is the one using as shown by Jeppsson *et al.* (1999); Khadka *et al.* (2005). However, the RAPD markers are not universal, but could be used for a species (Khadka *et al*., 2005) or a variety of hybrid offspring (Jeppsson *et al*., 1999), or a plant group as our research, because of the limits of male genotypes available in a species, varieties or a genus. According to our research and better, we have the RAPD-male related marker for two *Nepenthes*, *N. gracilis* and *N. mirabilis*. It is assumed to be a male marker for the genus *Nepenthes*. Also, the results indicate evidence that *Nepenthes* gender is genetically determined. It could be a powerful tool for selecting the male parent to be used in crosses and also could be used for paternity verification in progeny. Generally, male and female cultivars producing are more quality criteria to be met in female cultivars (Jeppsson *et al*., 1999). Therefore, the selection of sex needed at an early stage in the evaluation process could be saved much of work, money and time.

Male sex-linked genetic marker may not only be useful in breeding programs, but would also allow the understanding of the genetic and molecular basis dioecism in *Nepenthes*. Really, a diversity pitcher characters are more interesting than sexes, but, male pitcher plants is indeed related in producing hybrid offspring with attractive pitcher characters.

Obtaining young unknown *Nepenthes* species from a wild or a market, species identification is the first doing. The next important step is sex determination due to *Nepenthes* species are dioecious plants. One RAPD primer was produced a band, ranging in size about 750 bp, male-related marker appearing in both *N. mirabilis* and *N. gracilis*. Actually, it is very difficult to produce reproducible RAPD band, but the male-related marker primer is very specific possessing high resolution. It shows that the primer can be used for sex determination in any *Nepenthes* species making a few expense, a short time and simplified method.

After species identification and sex determination, our research is performing related to their interesting genes and differences in male and female pitcher plants.

REFERENCES

Avise, J.C., 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Chaveerach, A., H. Kunitake, S. Nuchadomrong, N. Sattayasai and H. Komutsu, 2002. RAPD patterns as a useful tool to differentiate Thai *Piper* from Morphologically alike Japanese *Piper*. Science Asia, 28: 221-226.

Chaveerach, A., A. Tanomtong, R. Sudmoon and T. Tanee, 2006. Genetic diversity among geographically separated populations of *Nepenthes mirabilis*. Biologia, Bratislava, 61: 295-298.

Clarke, C., 2002. A Guide to the Pitcher Plants of Peninsula Malaysia. Borneo: Natural History Publications.

Durand, R. and B. Durand, 1990. Sexual determination and sexual differentiation. Crit. Rev. Plant Sci., 9: 295-316.

Henderson, M.R., 1974. Malayan wild flowers, Dicotyledons: Nepenthaceae. Kuala Lumpur: The Malayan Nature Society.

Hooker, J.D., 1885. Nepenthaceae. Flora of British India Vol. 5. London: L. Reeve and Co., pp: 68-70.

Irish, E.E. and T. Nelson, 1989. Sex determination in monoecious and dioecious plants. Plant Cell, 1: 737-744.

Jeppsson, N., I.V. Bartish and H.A. Persson, 1999. DNA Analysis as a Tool in Sea Buckthon Breeding. In: Perspectives on New Crops and New Uses. Janick, J. (Ed.), Alexandria, VA: ASHS Press, pp: 338-341.

Khadka, D.K., A. Nejidat, M. Tal and A. Golan-Goldhirsh, 2005. Molecular characterization of a gender-linked DNA marker and a related gene in *Mercurialis annua* L. Planta, 222: 1063-1070.

Moran, J.A., 1996. Pitcher dimorphism, prey composition and the mechanisms of prey attraction in the pitcher plant *Nepenthes rafflesiana* in Borneo. Ecology, 84: 515-525.

Nybom, H. and H.K. Hall, 1991. Mini-satellite DNA fingerprints can distinguish *Robus* cultivars and estimate their degree of relatedness. Euphytica, 53: 107-114.

Ridley, H.N., 1967. Nepenthaceae. The Flora of the Malay Peninsula. London: L. Reeve and Co.

Shivas, R.G., 1984. Pitcher plants of the Peninsular Malaysia and Singapore. Maruzen: Koon Wah Printing Pte Ltd.

Smittinand, T., 1980. Thai Plant Names (Botanical Names-Vernacular Names). 2nd Edn., Bangkok: Funny Publishing.

Soltis, D.E. and P.S. Soltis, 1990. Isozymes in Plant Biology. London: Chapman and Hall.

Weier, T.E., C.R. Stocking, M.G. Barbour and T.L. Rost, 1982. Botany. New York: John Wiley and Sons.

Welsh, J., R.J. Honeycutt, M. McClelland and B.W.S. Sobral, 1991. Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). Theor. Applied Genet., 82: 473-476.

Wolfe, A.D., Q.Y. Xiang and S.R. Kephart, 1998. Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). Proceeding of Sciences USA., 94: 5112-5115.