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The Occurrence of Hybrid in *Nepenthes hookeriana* Lindl. from Central Kalimantan can be Detected by RAPD and ISSR Markers

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***Nepenthes* spp. (Nepenthaceae) is one of the most popular ornamental plants in Southeast Asia. There are 97 species of *Nepenthes* to which 64 are found in Indonesia with the center of its diversity located in Borneo. *N. x hookeriana* was hypothesised to be a natural hybrid between *N. ampullaria* and *N. rafflesiana* on the basis of morphological characters. Several variants of each species were also known. This present study aimed to detect the occurrence of hybrid within *N. x hookeriana* 'spotted' and 'green' variant using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR). Five RAPD primers and three ISSR primers were used to amplify total DNA genome and produced 83 polymorphic bands ranging in size from 300-1700 bp. Clustering analysis was performed based on RAPD and ISSR profiles using the UPGMA method. The genetic similarity of the combined markers range between 0.30-0.75 indicating a narrow range of genetic similarity among the accessions. Results from cluster analyses suggested that *N. x hookeriana* was indeed a hybrid between *N. ampullaria* and *N. Rafflesiana,* however it was genetically more similar to *N. raflessiana*.**

Key words: Hybrid, ISSR, *Nepenthes,* RAPD

INTRODUCTION

Nepenthes spp. (Nepenthaceae), locally known as Kantong Semar, has become a popular ornamental plant due to its unique behavior for being a carnivorous plant. *Nepenthes* was distributed from Madagascar, South East Asia, Southern China, Queensland, New Caledonia, New Guinea to Seychelles (Clarke 2001). It is mainly distributed in the wet tropics (Phillipps & Lamb 1996), with Borneo being the center of its diversity (Clarke 1997). To date, there are 97 species of *Nepenthes* (Mansur & Brearley 2008) to which 64 are found in Indonesia (Mansur 2006).

N. x hookeriana was hypothesized to be a natural hybrid between *N. ampullaria* and *N. rafflesiana* (Clarke 1997, 2001, 2004) on the basis of morphological characters. The shape of the lower pitcher of *N. ampullaria* is ovoid to urceolate with a large descending peristome and a small, ribbon-like lid (Figure 1a,b). While the shape of lower pitcher of *N. rafflesiana* is bulbous, the third upper part is cylindric with a large heart-shaped or ovoid lid. The peristome is oblique and tapering to the neck (Figure 1c). The shape of the lower pitcher of *N. x hookeriana* is somewhat a mixture between the putative parental species. The peristome is expanded but never overhangs the outer side of the lip, and the lid is wider than those of the *N. ampullaria*, but narrower than *N. rafflesiana* (Figure 1d,e). The pitcher is ovoid, similar to those of *N. ampullaria*, but is taller and somewhat thinner (Clarke 1997).

Thus, the derived characters from *N. ampullaria* were the shape of the pitcher and peristome, while the acquired character from *N. rafflesiana* was the large lid. Several variations of color and motifs were observed in *N. ampullaria* from Kalimantan, *i.e.* green, green with red lip, red, green spotted, and green spotted with red lip. Variations of colors and motifs recorded in *N. rafflesiana* were black spotted, red spotted and black reddish. While variations of colors and motifs found in *N. x hookeriana* were combinations of both parents, *i.e.* green, green spotted with red lip, and red blackish. *N. x hookeriana* occupied the same habitat as their putative parents, peat swamps forest and heath forest (Mansur 2007, 2008, 2010).

However, the evidence of a hybrid revealed from the morphological characters would also be supported by evidence from genetic features which have yet been discovered. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than any other markers. RAPD marker is a random fragment amplification technique, which is based on the random amplification of DNA fragments using single arbitrary primers. They have been widely used for genotyping plant species (Jimenez *et al.* 2002; Chakrabarti *et al.* 2006; Dnyaneshwar *et al.* 2006; Keller-Przyby³kowicz *et al.* 2006), evaluation of genetic relationship (Upadhyay *et al.* 2004; Goh *et al.* 2005; Oktavia *et al.* 2011) and genetic variation (Martin *et al.* 2002; Ferriol *et al.* 2003; Fan *et al.* 2004; Adetula 2006; Guo *et al.* 2007; Jain *et al.* 2007). The main advantages of this marker include rapid and cost-efficient in terms of operational aspects. Inter-simple sequence repeats (ISSR) is also a PCR-based technique, involving amplification of the region between two identical microsatellite repeats within the genome (Zietkiewicz *et al.* 1994). The main

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Figure 1. a. Morphology of *N. ampullaria* 'green', b. *N. Ampullaria* 'spotted', c. *N. Raflessiana*, d. *N. x hookeriana* 'green', e. *N. x hookeriana* 'spotted. P: pitcher, Ld: lid, Lp: lip.

advantage it is the capability of analyzing multiple loci in a single reaction. ISSR has been successfully utilized in assessing genetic diversity and relationships (Liu *et al.* 2006; Isshiki *et al.* 2008; Rucinska & Puchalski 2010) and genotype identification (Mattioni *et al.* 2002; Fracaro & Echeverrigaray 2006).

The objective of this present study was to investigate genetic variations among 29 accessions of *Nepenthes* using RAPD and ISSR markers. This would aid in detecting occurrences of hybrid within *N. x hookeriana* 'spotted' and 'green' from its putative parental species, *N. ampullaria* and *N. rafflesiana*.

MATERIALS AND METHODS

Plant Materials. Twenty nine samples of *Nepenthes* consisted of *N. x hookeriana* 'green' (6 accessions), *N. ampullaria* 'spotted' (6 accessions), *N. x hookeriana* '*spotted*' (6 accessions), *N. ampullaria* 'green' (5 accessions), and *N. rafflesiana* (6 accessions) were collected from Central Kalimantan. Samples were collected as dried leaves stored in silica gel.

Extraction of Total DNA Genome. Total DNA genome was extracted from dried leaves using modified CTAB (Doyle & Doyle 1990) by addition of RNAse 200 µg/ml. The total DNA genome was analyzed on 0.7% agarose gel electrophoresis in 1X TAE buffer at 100 Volt for 30 min, followed by ethidium bromide staining before photographed using gel documentation system (Atto Bioinstrument).

PCR Amplification. PCR amplification for RAPD and ISSR was performed in Takara thermocycler. Five RAPD primers (OPA 7, OPA 9E, OPB 10E, OPN 12, OPN 18E) and three ISSR primers (UBC 814 ,UBC826, UBC 834) were used to amplify total DNA genome. Three of the RAPD primers used were modified by adding 2 nucleotides in their 5 termini (Table 1). Amplifications were perfomed in 15 µl reaction volume containing a final concentration of 1x PCR *Green Master Mix* (Promega), 2 µM primer (Operon Technology Ltd.), and ~10 ng of DNA *template*.

The PCR condition for RAPD followed Williams *et al.* (1990), *i.e.* initial denaturation at 94 °C for 2 min, followed by 45 cycles of denaturation (94 °C for 1 min), annealing (36 °C for 1 min) and extension (72 °C for 2 min). These cycles were terminated by a final extension step at 72 °C for 2 min. The PCR condition of the ISSR amplification was initiated by a pre-denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C for 1 min), annealing (50 °C for 45 second) and extension (72 °C for 2 min). The cycles was finalised by an extenstion phase at 72 °C for 5 min.

Amplified products were separated in 2% agorose gel in 1X TAE buffer at 50 Volt for 120 min. The gels were stained with 0.5 µg/ml ethidium bromide solution, visualised and photographed using gel documentation system (Atto Bioinstrument). The PCR reactions were done twice to ensure the reproducibility and consistency of the PCR products.

Data Analysis. Both RAPD and ISSR bands were scored manually based on the profiles obtained from gel electrophoresis photos, as present (1) or absent (0), each of which was treated as a putative locus. This study used only bands that existed between 300-1700 bp. Generally, bands below 300 bp were inconsistent, while bands above 1700 bp could not be well separated during electrophoresis. Data analysis was performed using NTSYS-pc (Numerical Taxonomy System, version 2.02i, Rohlf 1998). The *Similarity for qualitative data* (SIMQUAL) program was used to calculate the Jaccard's similarity coefficient, a common estimator of genetic identity. Similarity matrices were utilised to construct the unweighted pair group method with aritmethical average (UPGMA) dendrograms. The same analysis described above was performed for each RAPD, ISSR and the combined data set. Finally, a principal coordinate analysis (PCO) was performed in order to highlight the resolving power of the ordination.

RESULTS

RAPD Analysis. Amplifications of genomic DNA of the 29 accessions using five primers yielded 53 fragments that could be scored. The number of amplified fragments ranging from 8 (OPA 7 and OPA 9E) to 13 (OPB 10E and OPN 18), with an average of 10.6 polymorphic fragments per primer whose size varied from 300 (all primers) to 1700 pb (OPB 10E) (Table 1). Common bands that existed in all accessions were OPA 7 at 900 bp, OPN 12 at 1000 bp, OPN 18E at 500 and 1500 bp. However, few bands only existed within certain accessions (Table 1).

A dendrogram based on UPGMA analysis grouped the 29 accessions into two main clusters (A and B), with Jaccard's similarity coefficient ranging from 0.27 to 0.73 (Figure 2). This implied that around 50% genetic similarity were shared among the accessions. Cluster A (coef. 0.33) was comprised of a minority of *N. x hookeriana* "spotted" and a majority of *N. rafflesiana*. Cluster B consisted of two sub-clusters C and D that consisted of *N. ampullaria* and *N. x hookeriana* members and a single lineage of *N. ampullaria* 'spotted' respectively.

ISSR Analysis. PCR products of ISSR amplifications could only be obtained on 21 accessions, and yielded 30 fragments that could be scored. The number of amplified fragments ranged from nine (UBC 834) to 11 (UBC 826), with an average of 10 polymorphic fragments per primer whose size varied from 300 (UBC 834) to 1700 pb (UBC 826) (Table 1). Common bands that existed in all accessions were UBC 814 at 600 and 1500 bp, UBC 826 at 800 and 1500 bp, UBC 834 at 300 and 500 bp. Nevertheless, few bands were found only in certain accessions (Table 1).

A dendrogram based on UPGMA analysis showed that all accessions did not group into distinct clusters, but formed gradual groupings. Members of *N. ampullaria* tend to be located at the basal clusters as single lineage, followed by *N. rafflesiana*, and *N. x hookeriana* at the terminal cluster (Figure 3). However, there was one accession of *N. hookeriana* 'spotted' that formed a group together with *N. ampullaria* 'spotted' located between *N. x hookeriana* and *N. rafflesiana*'s clusters.

Combined RAPD and ISSR Analysis. When the two data matrices were combined, UPGMA dendrogram showed a rather different result. Three clusters (A, B, C) were formed with Jaccard's similarity coefficient ranging from 0.30 to 0.75 (Figure 4). Cluster A consisted mainly of *N. x hookeriana*. *N. x hookeriana* 'spotted' tended to located within internal branches of *N. x hookeriana* and *N. rafflesiana* clusters. *N. rafflesiana* formed a group with ~47% similarity, while *N. ampullaria* 'green' formed a group with ~50% similarity.

PCO analysis resulted in four groups (A, B, C, D, Figure 5) with group A, B, C corresponding to the groupings in the cluster dendrogram. Group D contained accessions that corresponded to cluster B (Figure 4).

Table 1. RAPD and ISSR primers used, polymorphic bands and their distribution in each species/variant. Bold type letters were additional nucleotides. 1: *N. x hookeriana* 'green', 2: *N. ampullaria* 'spotted', 3: *N. x hookeriana* 'spotted', 4: *N. ampullaria* 'green', 5: *N. rafflesiana*

Primer's name	DNA sequence (52-32)	Total and polymorphic bands	Size range (bp)	Common bands (bp)	Unique bands observed in each variant (bp)				
					1	2	3	4	5
OPA 7	GAA ACG GGT G	8 (8)	300-1100	800	-	-	-	900	-
OPA 9E	**TT**GGGTAACGCC	8 (8)	300-1100	650	-	-	600	-	-
OPB 10E	**CA**CTGCTGGGAC	13 (13)	300-1700	700	-	400	-		
OPN 12	CAC AGA CAC C	13 (13)	300-1400	950	-	-	-	-	-
OPN 18E	**AA**GGTGAGGTCA	11 (11)	300-1400	500; 1400	-	-	-	-	-
UBC 814	CTCTCTCTCTCTCTCTA	10 (10)	500-1500	600; 1500	1100	-	500;1400	-	1000
UBC 826	ACACACACACACACACC	11 (11)	350-1700	800; 1500	-	-	750	-	-
UBC 834	AGAGAGAGAGAGAGAGYC	9 (8)	300-1500	300; 500	1400	500	-	-	400;1500

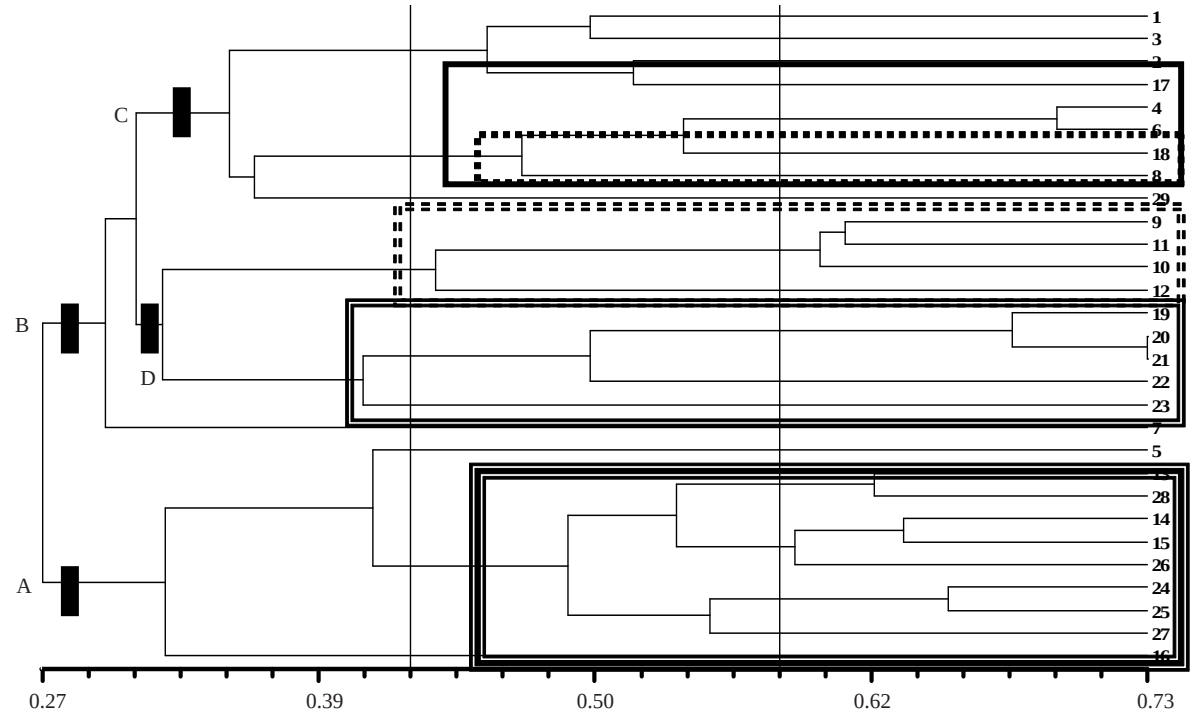

Figure 2. Cluster diagram based on Jaccard coeffient of similarity in 29 accessions of *Nepenthes* using RAPD marker. 1-6: *N. xhookeriana* 'green', 7-12: *N. ampullaria* 'spotted', 13-18: *N. xhookeriana* 'spotted', 19-23: *N. ampullaria* 'green', 24-29: *M. rafflesiana.*. Single-line solid box: *N. hookeriana*'green', single-line dotted box: *N. hookeriana* 'spotted', double-lines solid box: *N. ampullaria* 'green', double-lines dotted box: *N. ampullaria* 'spotted', triple-lines solid box: *N. rafflesiana*. Vertical dotted lines: reference lines.

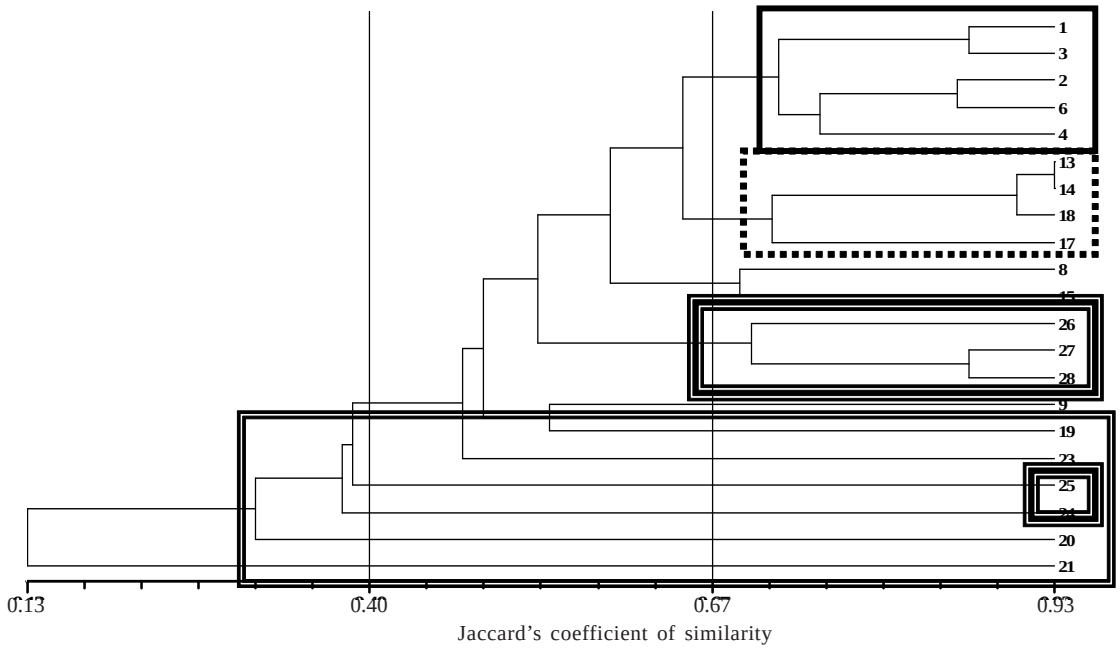

Figure 3. Cluster diagram based on Jaccard coeffient of similarity in 21 accessions of *Nepenthes* using ISSR marker. 1-6: *N. xhookeriana* 'green', 7-12: *N. ampullaria* 'spotted', 13-18: *N. xhookeriana* 'spotted', 19-23: *N. ampullaria* 'green', 24-29: *M. rafflesiana.* Single-line solid box: *N. hookeriana*'green', single-line dotted box: *N. hookeriana* 'spotted', double-lines solid box: *N. ampullaria* 'green', triple-lines solid box: *N. rafflesiana*. Vertical dotted lines: reference lines.

DISCUSSION

Results from PCR amplification indicated that each RAPD and ISSR primer could generate a polymorphism. The five RAPD primers and three ISSR primers generated 83 polymorphic bands that were evenly distributed among the samples with exception of a few bands that were exclusively found in some individuals (Table 1). The difference in the level of polymorphism detected among the markers could be attributed to the type of region

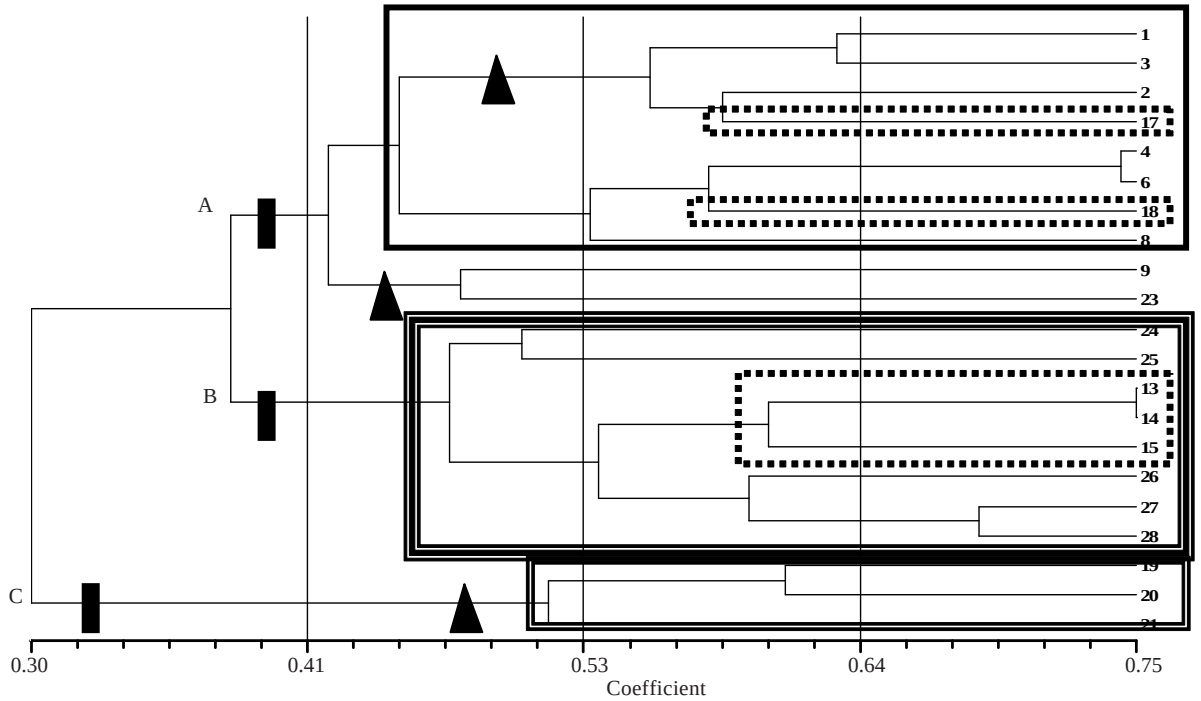

Figure 4. Cluster diagram based on Jaccard coeffient of similarity in 21 accessions of *Nepenthes* using RAPD and ISSR markers. 1-6: *N. xhookeriana* 'green', 7-12: *N. ampullaria* 'spotted', 13-18: *N. xhookeriana* 'spotted', 19-23: *N. ampullaria* 'green', 24-29: *M. rafflesiana.* Single-line solid box: *N. hookeriana*'green', single-line dotted box: *N. hookeriana* 'spotted', double-lines solid box: *N. ampullaria* 'green', triple-lines solid box: *N. rafflesiana*. Vertical dotted lines: reference lines. Triangle: clusters that correspond to the grouping in PCA diagram.

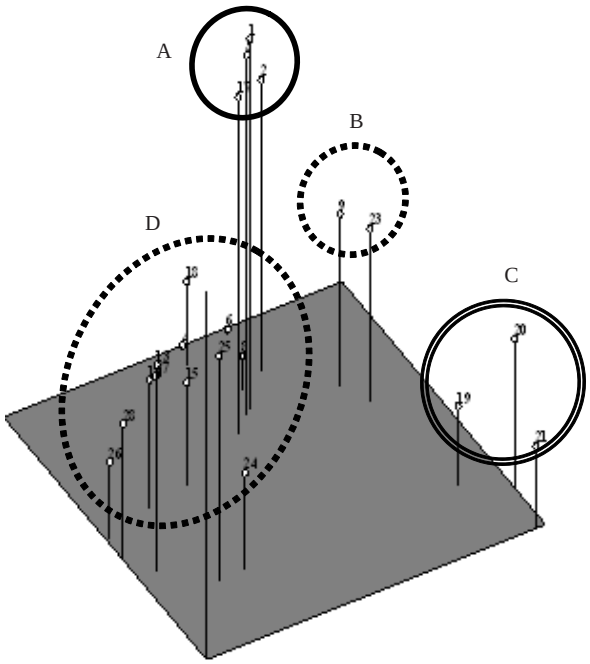

Figure 5. Three-dimensional plot of principal coordinate analysis of 21 accesions of *Nepenthes*. The numbers plotted represent individual sampel and corresponds to notes in Table 1 Single solid and double-lines circles are corresponding to the grouping in the cluster diagram.

amplified in each case, since the ISSR markers amplified relatively conserved regions present among the microsatellites sequences, whereas the RAPD markers amplified random regions (Zietkiewicz *et al.* 1994). ISSR was thought to have a higher capacity to reveal polymorphism than RAPD (Zietkiewicz *et al.* 1994). This study, however, showed that ISSR produced less polymorphic bands than that of RAPD. This may be caused by the use of less numbers of ISSR primers. Souframanien and Gopalakrishna (2004) suggested that the ability to resolve genetic variation among different genotype maybe more directly related to the number of polymorphism detected within each marker technique rather than a function of which technique is employed. Genetic variations found in this study was therefore implied from differences on RAPD and ISSR profiles in all accessions.

Clustering analysis using three data sets resulted in rather different results. RAPD analysis implied that *N. x hookeriana* was genetically more similar to *N. ampullaria* than to *N. rafflesiana,* while both ISSR and RAPD-ISSR analyses suggested that *N. x hookeriana* was genetically more similar to *N. rafflesiana* than to *N. ampullaria.* Similarity of results obtained from ISSR and ISSR+RAPD was also observed by Souframanien and Gopalakrishna (2004). Differences in the clustering of genotypes using RAPD and ISSR may be due to the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among accessions (Loarce *et al.* 1996). In addition, the putatively similar bands originated by RAPD analysis in different accessions were not necessarily homologous, although they may share the same size.

Clustering analysis of both data set resulted in three main clusters, each containing *N. ampullaria* group, *N. x hookeriana* group and *N. x hookeriana-N. raflesiana*

group. *N. ampullaria* formed a group inclusively, which did not contain any member of *N. x* hookeriana. Compared to other species of *Nepenthes*, *N. ampullaria* had a distinct character for having a pitcher that was almost ovoid, its stem often formed a rossette pitcher which spread carpet-like over the soil surface. This species was also known as a vegetarian pitcher because they feed on litter fall. Unlike any other *Nepenthes*, their peristome and lid did not contain any nectar glands, therefore no insects were attracted to visit their pitcher (Clarke & Lee 2004). These unique morphological characters were therefore confirmed by RAPD analysis by having profiles that were genetically distant from the other clusters.

It is interesting to note that most members of *N. x hookeriana* "spotted" were forming groups together with *N. rafflesiana* with a similarity coeffient of 0.60 (Figure 5). Marsolais *et al.* (1993) suggested that the range of 0.50 using RAPD could imply the occurence of interspecific hybrid, while a range between 0.61-0.99 could suggest genetic similarity at the spesies level in Lilac, while interspecific hybrid in *Mentha spicata* and *M. arvensis* shared a 56% and a 49% similarity to the parents (Shasany *et al.* 2005). Hence, genetically, *N. x hookeriana* "spotted" was more similar to *N. rafflesiana* as one of their parental species. This may have occured because the samples of *N. x hookeriana* were obtained from hybrid between male *N. rafflesiana* with female *N. ampullaria* 'green',While *N. x hookeriana* 'green' was hypothesed to be a hybrid between male *N. ampullaria* 'green' and female *N. rafflesiana*. Thus, the variation of colors and motifs were determined by male *Nepenthes*.

Cluster A (Figure 5) contained only members of *N. x hookeriana* 'green' and the 'spotted' variant (~0.45 similarity). Although the spotted variant is included, they were genetically distinct from the green variant. This may indicate that *N. x hookeriana* 'green' may have separate lineages and may potentially become a separate genetic entity from both putative parents, *N. ampullaria* and *N. rafflesiana*.

The result of PCO, to a certain extent, was comparable to the cluster analysis (Figure 6). Group A, B, C appeared to be distinct from other accesions in the PCO. Otherwise, the remaining components that were grouped in PCO may have contributed to the total variation corresponding to the polymorphic loci.

We concluded that the results from our study supported the morphological data in that *N. x hookeriana* was indeed a hybrid between *N. ampullaria* and *N. rafflesiana* but genetically more similar to *N. rafflesiana*. We also showed that RAPD and ISSR marker could be sufficiently used to detect the occurence of a hybrid in *N. x hookeriana*. Inclusion of more samples representing all observed variants into the analysis and the use of other markers could provide a higher resolution to the analysis.

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