

# Analysis of Natural Hybrid Kantung Semar (*Nepenthes*) in Bukik Taratak West Sumatra with RAPD Technique

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**Abstract.** Three types of *Nepenthes*, namely *N. ampullaria*, *N. mirabilis* and *N. gracilis*, which live sympatrically in Bukit Taratak, are thought to produce hybrids that have been studied morphologically, but molecular analysis is needed to confirm the hybrid. The aim of this study was to prove natural hybrid *Nepenthes*'s with RAPD technique and to find out natural hybrid relationship among *Nepenthes*'s with both parents. The method used is RAPD using OPA 15, OPK 16, OPK 19 and OPP 15 primers. The hybrid relationship uses PCO analysis processed with PAST 2.10 program. This study proves the existence of natural hybrid types between *N. ampullaria* × *N. mirabilis* and *N. ampullaria* × *N. gracilis*. The natural hybrid relationship of *N. ampullaria* × *N. mirabilis* is closer to *N. mirabilis*, while the natural hybrid *N. ampullaria* × *N. gracilis* is closer to *N. Ampullaria*.

**Keywords :** *Nepenthes*, RAPD, Natural, Hybrid

## 1. Introduction

*Nepenthes* is a unique plant [1-6]. The uniqueness and distinctiveness of *Nepenthes* is found in the character of the bag which is a modification of the end of the leaf, so that this plant is called the *Pitcher Plant*. *Nepenthes* have varying in shapes and colors. In Indonesia, in general this plant is better known as the Kantung Semar, but in some areas *Nepenthes* is also called by a local name, including *kettles*, *monkey pouches* and *kuran-kuran*. In West Sumatra, *Nepenthes* is known as *Saluang antu*, *Kumbuak-Kumbuak* and *Katidiang Baruak* [7].

One of the distribution areas *Nepenthes* in West Sumatra is Taratak Hill. Taratak Hill is a part of Koto Taratak Village, Pesisir Selatan Regency. In the Taratak Hill region, three types *Nepenthes* founded, namely *N. ampullaria*, *N. mirabilis* and *N. gracilis*. Based on preliminary observations in the field, these three species *Nepenthes* live sympatrically in the area and are found to flower almost at all times [7,8]. Sympatric populations and their ability to flower almost all the time provide greater possibilities for cross breeding and natural hybrid formation in the area. In this area, individuals *Nepenthes* were found to have a mix character among the species *Nepenthes* in the area. These individuals are thought to be natural hybrids between *N. ampullaria* × *N. mirabilis*; *N. ampullaria* × *N. gracilis* and *N. gracilis* × *N. mirabilis* [8].

Analysis of a natural hybrid can be done, among others, by using morphological, cytological and molecular characters [9]. Morphological studies of natural hybrids in the Taratak

Hill found that there were three hybrids in the Taratak Hill, namely hybrid *N. ampullaria* × *N. mirabilis*; *N. ampullaria* × *N. gracilis* and *N. gracilis* × *N. mirabilis* [8]. Hybrid testing with morphological character, at this time is not enough, because identification based on morphological characters has a very wide variation and can still be influenced by environmental factors and can not be done in juvenile or tillering [10] so it will be better to confirmed again with molecular testing.

RAPD technique is the simplest and fastest technique because it only requires amplification of the target DNA with PCR, without having to know the target DNA sequence. RAPD markers can be used in identification of varieties or strains, phylogenetic studies and genetic mapping [11]. RAPD is a technique that can be used to confirm natural hybrids [12].

## 2. Experimental Section

### 2.1. Tools and Materials

Tools needed in a sample collection are GPS (Global Positioning System) navigation tools, cutting tools and plastic collections. The material used for sample collection is young leaves (shoots) of plants and silica gel as sample preservatives. Equipment needed for the isolation of DNA, namely scissors, *Mortar and Pestle*, *ependorf* 2 mL and 1.5 mL of sterile, spatula, waterbath, 10-200 mL pipette, 100-1000 mL pipette, vortex, *Centrifuge* max. 14,000 rpm, *chemical hood*, *ependorf tube* stand (test tube rack), 37°C incubator, evaporator and refrigerator.

The tools used for amplification and electrophoresis are 2-10 µL pipette, PCR machine, electrophoresis tub, *power supply*, *comb* 8 slot, *gel tray* (gel mold). Gel inhibitors and documentation devices. Materials needed for DNA extraction are extraction buffers, 3M Na-acetate stored in cold conditions, chloroform: isoamylalkohol, cold absolute ethanol, 70% ethanol, and TE-RNase. The materials needed for amplification are 10x PCR Buffer, MgCl<sub>2</sub> (25 mM), dNTPs mix (10 mM), Primary (OPA 15, OPK 16, OPK 19 and OPP 15), *Taq* DNA Polymerase, ddH<sub>2</sub>O and mold DNA (10 ng / ul). The materials needed for electrophoresis are agarose, 1x TBE buffer, and ethidium bromide as coloring material.

### 2.2. Methods used

#### 2.2.1. Sample Collection in the Field

The sampling position was recorded using the GPS (Global Positioning System) navigation tool. A sample of putative hybrid leaves *Nepenthes ampullaria* × *N. mirabilis*, *N. ampullaria* × *N. gracilis*, *N. ampullaria*, *N. mirabilis* and *N. gracilis*, and male and female individuals from their respective parents (*N. ampullaria*, *N. mirabilis* and *N. gracilis*), were taken and stored in a collection of plastic filled with silica gel. Samples in silica gel are stored in a dry place, if silica gel has been pink, replaced with new silica gel, until the sample is used.

#### 2.2.2. Isolation and amplification of DNA

DNA isolated from young leaves of putative hybrid and both parents. DNA isolation was carried out by modification of CTAB method, DNA isolation protocol developed by Doyle and Doyle [13]. DNA amplification was carried out according to Williams, *et al.* [14]. This study used 4 RAPD primers obtained from the BB Biogen Molecular Biology Laboratory, Bogor, namely OPA-15, OPK-16, OPK-19 and OPP-15. Primer refers to research on molecular analysis *Nepenthes*

with RAPD and RAPD Hybrid Analysis that has been done previously [15,16,17]. DNA electrophoresis was carried out based on methods found by Smithies with several modifications [18].

### 2.2.3. Natural Hybrid Analysis

Data were analyzed descriptively where hybrid assertion was based on the specific bands of both parents and their inheritance in natural hybrids. Analysis of natural hybrid kinship with both parents was carried out using cluster analysis and PCO (*Principle Coordinate*), where DNA bands were scored 1 for the presence of bands, and 0 for the absence of bands. Cluster analysis is done by calculating the value of genetic similarity based on the Jaccard similarity coefficient with the output in the form of dendrogram carried out by the method *Paired Group*. In addition to the cluster analysis, analysis was conducted *Principle Coordinate (PCO)* to determine the pattern of hybrid genetic variations with both parents. Cluster analysis and PCO were processed using the Analysis program using the *Paleontological Statistics (PAST)* program 2.10.

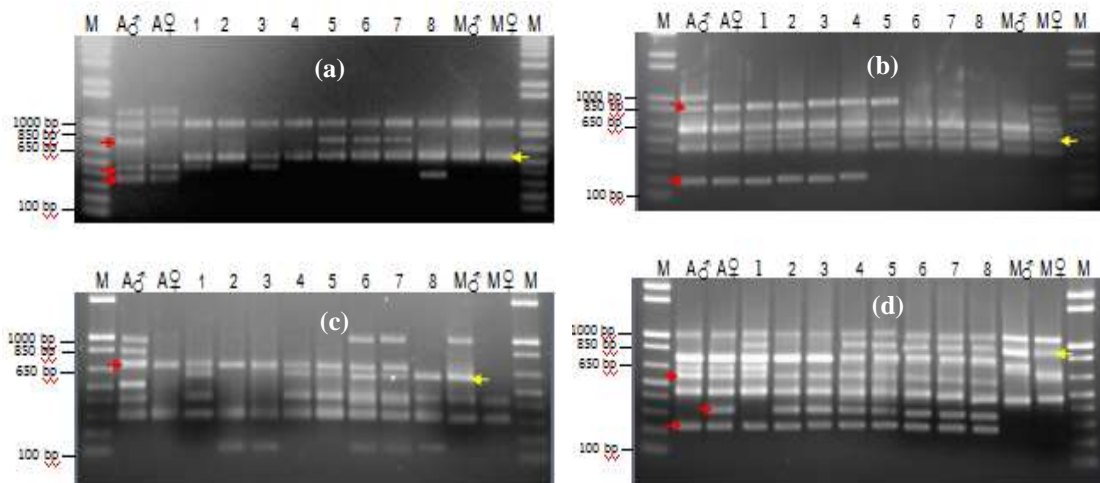
## 3. Results and Discussion

Natural hybrids *Nepenthes* found in Taratak Hill Pesisir Selatan are natural hybrids between species *N. ampullaria* and *N. mirabilis*; *N. ampullaria* and *N. gracilis*. Previous studies which reported that the natural hybrid of *N. ampullaria* x *N. mirabilis* was determined by looking at the character of the edge of the leaf which partly had coarse hairs that were rare and long and some had short, dense feathers, which were the characters of the mixture from the edge of the leaf *N. ampullaria* which is short-haired and tight with the edges of the leaves *N. mirabilis* which are rough and long hair which is rare and long. The lower sac is in the form of a urceolate like *N. ampullaria* but in the upper part of the tube, which pushes up to reach the lip of the sac. The character of the sac lips has a shape between the two parents, such as the wide and flat *N. mirabilis* lip shape of plus the shape of the sac *N. ampullaria* which curves long into the inside of the bag [19].

The natural hybrid form of *N. ampullaria* x *N. gracilis* is a form between the two parents, according to previous studies which found that the natural hybrid *N. ampullaria* x *N. gracilis* was determined by looking at the character of the pocket lips wider than the lips of *N. gracilis*, but narrower from the lips of *N. ampullaria*. The oval to almost round hybrid pocket cover, which is a mixed character of lanceate *N. ampullaria* the linear-sac lid and closes the sac *N. gracilis* orbicularis-ovate. The shape of a hybrid bag like *N. gracilis* with a few spotting [8].

### Putative hybrid *N. ampullaria* x *N. mirabilis*

RAPD technique using OPA 15, OPK 16, OPK 19 and OPP 15 primers showed polymorphisms in putative hybrids and both parents (Figure 1). The four primers produced 29 bands measuring 200-1100 bp. 23 (79.32%) of them were polymorphic and only 6 bands were found in monomorphic. The primary sequence of polymorphic was the highest OPK 16 (87.5%), followed by OPA 15 (83.33%), OPP 15 (75%) and OPK 19 (71, 42%). Four primers, 12 specific bands were obtained *N. ampullaria* and 5 specific bands from *N. mirabilis* (table 1).



**Figure 1.** Pattern of natural hybrid RAPD bands *N. ampullaria* × *N. mirabilis* and both parents; a) OPA 15; (b) OPK 16; (c) OPK 19; (d) OPP 15;

**Table 1.** The presence of the hybrid ribbon *N. ampullaria* x *N.mirabilis* and two parents  
 Ket: (1-8) a natural hybrid individuals *ampullariaN.* x *N. mirabilis*

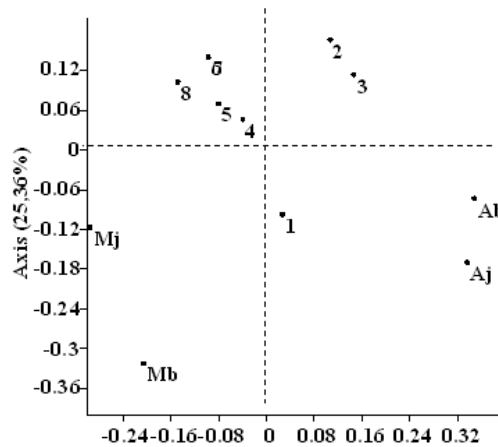
Primer RAPD	No pita (bp)	Pola Kehadiran Pita DNA												
		<i>N. ampullaria</i>		<i>N. ampullaria</i> x <i>N. mirabilis</i>								<i>N. mirabilis</i>		
		Jantan	Betina	1	2	3	4	5	6	7	8	Jantan	Betina	
OPA 15	I (350)	+	+	-	-	-	-	-	-	-	-	+	-	-
	II (450)	+	+	+	-	+	-	-	-	-	-	-	-	-
	III(550)	-	-	+	+	+	+	+	+	+	+	+	+	+
	IV(700)	+	-	-	+	-	-	+	+	+	-	-	-	-
	V(1000)	+	+	+	+	+	+	+	+	+	+	+	+	+
	VI(1100)	+	+	-	-	-	-	-	-	-	-	-	-	-
OPK 16	I (100)	-	-	-	+	+	-	-	+	+	+	-	-	
	II (300)	+	+	+	+	+	+	+	+	+	+	+	+	
	III(400)	+	-	+	-	-	+	+	+	+	+	+	+	
	IV(500)	+	-	-	-	-	-	-	-	-	-	-	-	
	V (600)	-	-	+	-	-	+	+	+	+	+	+	-	
	VI(700)	+	+	+	+	+	+	+	+	+	-	-	-	
	VII(850)	+	-	-	-	-	-	-	-	-	-	-	-	
	VIII(1000)	+	+	-	-	-	-	-	+	+	-	+	-	
OPK 19	I (200)	+	+	+	+	+	-	-	-	-	-	-	-	
	II (400)	+	+	+	+	+	+	+	+	+	+	+	+	
	III(500)	-	-	+	+	+	+	+	+	+	+	+	+	
	IV(600)	+	+	+	+	+	+	+	+	+	+	+	+	
	V (700)	-	-	-	-	-	-	-	-	-	-	-	+	
	VI(850)	+	+	+	+	+	+	-	-	-	-	-	+	

	VII(1000)	+	-	-	-	-	-	-	-	-	-	-
OPP 15	I (200)	+	+	+	+	+	+	+	+	+	-	-
	II (300)	-	+	-	+	+	+	+	+	+	-	-
	III(400)	+	+	+	+	+	+	+	+	+	+	+
	IV(550)	+	+	+	+	+	+	+	+	+	-	-
	V (650)	+	+	+	-	-	-	-	-	-	-	+
	VI(700)	+	+	+	+	+	+	+	+	+	+	-
	VII(850)	-	-	+	-	-	+	+	+	+	+	+
	VIII(1000)	+	+	+	+	+	+	+	+	+	+	+

The identification of hybrids confirmed by looking at specific bands in both types of parents which inherited by individual derivatives [20]. Ribbons may appear only in hybrids, but are not in both of parents, and bands may appear only in one parent, do not appear in hybrids and other parent [10]. This was found in this study, with Primer OPA 15, the 1100 bp band is a band that only appears on *N. ampullaria*. with OPK 16 there is also a band, which is only on *N. ampullaria* male, which is at 500 bp and 850 bp. In OPK 19, there is only 1 band which is only owned by *N. ampullaria* male, which is at 1000 bp, and the band is only owned by *N. mirabilis*, which is at 700 bp. *Crossing-over* that occurs during meiosis may cause the loss of important loci, so that markers that appear on the elders do not appear in the offspring. Also on OPK 16 primer, a band that only appears on hybrids, which is at 100 bp, is a unique band. This unique band appears in the offspring which may be a result of recombination, random mutation or segregation that occurs on meiotic chromosomes during hybrid formation [15].

**The Proximity among *N. ampullaria* x *N. Mirabilis* with parental.**

PCO analysis proves that some hybrids have proximity to the first type of parent and some hybrids have proximity to second type parents and some hybrids have a balanced proximity to both parents (figure 2).



**Figure 2.** PCO ordination plot on *N. ampullaria* × *N.mirabilis* hybridwith its parents.

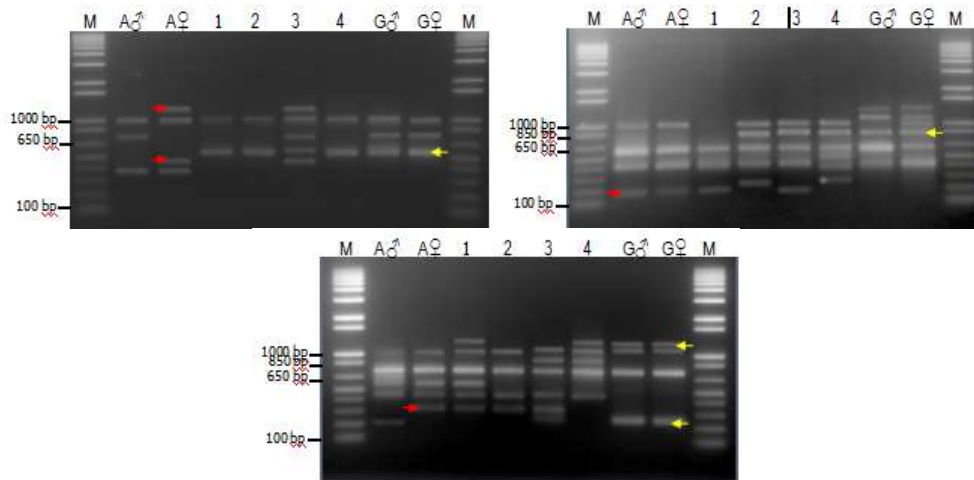
Ket: Aj; *N. ampullaria* male; Ab: *N.ampullaria* female, (1-8): hybrid;

Mj: *N. mirabilis* male; Mb: *N. mirabilis* Female

Analysis PCO result that 5 hybrids have proximity to *N. mirabilis* and 3 have proximity to *N. ampullaria*. Hybrids 1, 2 and 3 with the RAPD technique have a closer relationship with the elders of *N. ampullaria*, while hybrids 4, 5, 6, 7 and 8 are closer to the elders of *N. mirabilis*. But of course this assumption requires further research.

**Putative hybrid *N. ampullaria* x *N. gracilis***

Three primers that can provide good amplification results in identifying putative hybrids *N. ampullaria* x *N. gracilis*, namely OPA 15, OPK 19 and OPP 15 (figure 3). The three primers show polymorphisms in putative hybrids and both parents (table 2). The three primers produced 25 bands measuring 200-1400 bp and from the 25 bands, 20 (80.37%) of them were polymorphic and only 5 bands were found monomorphic. The primary sequence of the highest polymorphic was OPA 15 primer (83.33%), OPP 15 (80%) and OPK 19 (77, 77%).



**Figure 3.** The pattern of the hybrid RAPD band *N. ampullaria* × *N. gracilis* and both parents; (a) OPA 15; (b) OPK 19; (c) OPP 15

In this hybrid, from the three primers there were 6 bands which only appeared in natural hybrids, and were not found in parents, 2 bands in OPA 15 primer (450 bp, 1100 bp), 2 bands in OPK 19 (300 bp, 500 bp) and 2 bands in OPP 15 primer (300 bp, 850 bp). The three primers used produced 15 specific bands in the parents, 9 specific bands of *N. ampullaria* and 6 specific bands of *N. gracilis*.

**Table 2.** The presence of the hybrid ribbon *N. ampullaria* x *N. gracilis* and two parents Ket: (1-4) a natural hybrid individuals *ampullaria* *N.* x *N. gracilis*

RAPD Primers	No Pita (bp)	Pola Kehadiran Pita DNA							
		<i>N. ampullaria</i>		<i>N. ampullaria</i> x <i>N. gracilis</i>				<i>N. gracilis</i>	
		Jantan	Betina	1	2	3	4	Jantan	Betina
OPA 15	I (400)	+	+	-	-	-	-	-	-
	II (450)	-	+	-	-	+	-	-	-
	III(600)	-	-	+	+	+	+	+	+

OPK 19	IV(700)	+	-	-	-	+	-	+	+
	V(1000)	+	+	+	+	+	+	+	+
	VI(1100)	-	+	-	-	+	-	-	-
	I (200)	+	+	+	-	+	-	-	-
	II (300)	-	-	-	+	-	+	-	-
	III(400)	+	+	+	+	+	+	+	+
	IV(500)	-	-	-	-	-	+	-	-
	V(600)	+	+	+	+	+	+	+	+
	VI(850)	-	-	-	+	+	+	+	+
	VII(1000)	+	+	-	+	+	+	-	+
OPP 15	VIII(1100)	-	-	-	-	-	-	+	+
	IX(1400)	-	-	-	-	-	-	+	+
	I (200)	+	-	-	-	-	-	-	-
	II (250)	-	-	-	-	+	-	+	+
	III(300)	-	+	+	+	+	-	-	-
	IV(450)	+	+	+	+	+	+	-	-
	V(550)	+	+	+	-	-	-	-	-
	VI(650)	+	-	-	-	-	-	-	-
	VII(700)	+	+	+	+	+	+	+	+
	VIII(850)	-	-	-	-	+	+	-	-
IX(1000)	+	+	+	+	+	+	+	+	
X(1100)	-	-	+	-	-	+	+	+	

Three primers used gave better results for identified hybrid *N. ampullaria x N. mirabilis* than hibrid *N. ampullaria x N. gracilis*. This can be assumed due to errors in the introduction of hybrid *N. ampullaria x N. gracilis* visually with morphological characters. In this study the number of primers used has provided good results in analyzing hybrids, but it would be better if we use more primers, so it might be possible to produce more varied and stronger ribbon and polymorphic patterns in analyzing hybrids.hybrid research *Nepenthes* might get better and more accurate results when using more RAPD primers.

### The Proximity among *N. ampullaria x N. Mirabilis* with parental

PCO analysis proves that some hybrids have proximity to the first type of parent and some hybrids have proximity to the second type of parent and some hybrids have a balanced proximity to both parents.

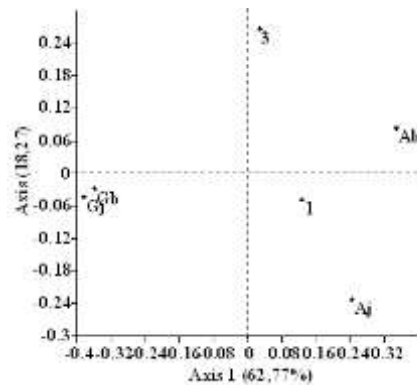


Figure 10. PCO ordination plot on *N. ampullaria* × *N. gracilis* hybrid with its parent  
 Ket: Aj; *N. ampullaria* male; Ab: *N. ampullaria* female, (1-4): hybrid;  
 Gj: *N. gracilis* male; Fig: *N. gracilis* females

From axis 1 it can be seen that hybrids 1 and 3 with the RAPD technique have a closer relationship with the elders of *N. ampullaria*. From axis 2 it can be seen that hybrid 1 is closer to *ampullaria* male and hybrid 3 is closer to *N. ampullaria* female. While we can assume that hybrid 1 has parental *N. ampullaria* male and *N. gracilis* female and hybrid 3 have parental *N. ampullaria* female and *N. gracilis* male. But of course this assumption requires further research.

#### 4. Conclusion

Based on the research conducted, it was concluded that the *Nepenthes* species found in Bukit Taratak Pesisir Selatan with the RAPD method proved to be true hybrids that occur naturally between *N. ampullaria* × *N. mirabilis* and *N. ampullaria* × *N. gracilis*. The natural hybrid relationship of *N. ampullaria* × *N. mirabilis* is closer to *N. mirabilis*, while the natural hybrid *N. ampullaria* × *N. gracilis* is closer to *N. ampullaria*.

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