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Genetic Variability Based on Randomly Amplified Polymorphic DNA in Kacip Fatimah (*Labisia pumila* Benth & Hook f) collected from Melaka and Negeri Sembilan States of Malaysia

Subhash J Bhore[1*], A H Nurul[2], and Farida H Shah[3]

[1]*Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong-Semeling Road, Bedong, 08100, Kedah Darul Aman, Malaysia*

[2]*Molecular Biology Division, Melaka Institute of Biotechnology, Lot 7, Melaka International Trade Center City, Ayer Keroh, 75450, Melaka, Malaysia*

[3]*Novel Plants Sdn. Bhd., Taman Rambai Harmoni, Off Jalan Tanjuag Minyak, 75260, Melaka, Malaysia*

ABSTRACT : In Malaysia, *Labisia pumila* Benth & Hook f, popularly known as 'Kacip Fatimah' has been used traditionally to treat various elements of the woman's health in Malay community. The objective of this study was to develop randomly amplified polymorphic DNA (RAPD) based DNA markers for the identification of *L. pumila* and to distinguish its three varieties from each other. Total DNA from nine accessions of *L. pumila* was extracted by CTAB method and polymerase chain reactions (PCR) were carried out to amplify the segments of DNA using different primers to develop DNA barcode using RAPD technique. To find out variety-specific DNA marker/s, twenty different 10-mer primer sequences with annealing temperature from 36-40 °C were evaluated in triplicate. Out of 20 random primers, two primers (OPA-1 and OPA-2/A10) were selected which produced reliable RAPD band patterns. To have DNA based handle, two RAPD amplification products were cloned and sequenced to determine the identity of the DNA. RAPD analysis using two random primers generated 72 discrete bands ranging in size 200 bp-3,000 bp. Fifty nine of these were polymorphic loci (82%) and thirteen were non-polymorphic loci (18%). A total of 32 bands polymorphic loci (72%) were amplified with primer OPA-1 and analyzed by cluster analysis and UPGMA (Unweighted Pair Group Method with Arithmetic) to present a dendogram depicting the degree of genetic relationship among nine accessions of *L. pumila*. Our results shows the reasonable genetic diversity among the *L. pumila* varieties and within varieties; and two RAPD marker sequences obtained could be used to identify *L. pumila* at species level.

Keywords : Biodiversity, DNA polymorphism, *Labisia pothoina*, Medicinal plants, RAPD

INTRODUCTION

Malaysia is the 12th most biodiverse nation in the world and ranks fourth in Asia with over 15,000 flowering plants and over 3,000 species of medicinal plants. From the 3,000 listed medicinal plants, only about 50 plants are used commercially and even less are being studied scientifically for their medicinal properties. Many more have yet to be catalogued through ethnobotanical research. *Labisia pumila* Benth & Hook f (*Labisia pothoina* Lindl) (Edwards's Bot. Reg. 31: t. 48. 1845; www.tropicos.org/Name/22002192) is one of the medicinal plants that have been exploited as traditional medicine and to develop various products such as drinks, extract based capsules and cocktail herbal products (Shahrim et al., 2006).

Labisia pumila popularly known as 'Kacip Fatimah' (in Malaysia) is a popular traditional herb and well recognized by Malay women for its medicinal value. Traditionally, the plant is boiled and the water extract is taken to stimulate and facilitate the childbirth and as a postpartum medicine. As stated by Ayida et al. (2007) it is widely believed that *L. pumila* (var. *alata*) extracts helps in firming and toning of abdominal muscles, breasts and tighten vaginal muscles, anti-dysmenorrhoea; cleansing and avoiding pain-

* Corresponding author: (E-mail) subhash@aimst.edu.my

ful or difficult menstruation, to treat dysentery, women's ailments associated with childbirth (Burkill and Haniff, 1965; Ayida et al., 2007), and to regain their energy and to delay fertility (Shahrim et al., 2006). Husniza et al. (2000) as cited in IMR (2002) showed that water extracts of the *L. pumila* produce a dose-response effect on the reproductive hormones of female rats, particularly on the estradiol and free testosterone levels. Other claimed traditional uses of *L. pumila* (var. *alata*) extracts include in treatment for rheumatism, indigestion, reducing menstruation pain, in treatment of haemorrhoids, to enhance the female appearance, and as a 'female aphrodisiac'(Fasihuddin, 1993).

Three varieties of *L. pumila* namely, *L. pumila* var. *alata*, *L. pumila* var. *pumila* and *L. pumila* var. *lanceolata* had been identified and found in Malaysia (Stone, 1994). It is believed that each variety commands a different use, and hence it is important to ensure that the right variety is used in each case (Fasihuddin, 1993). It is essential to differentiate these three varieties in terms of their physical and chemical characteristics as well as biological activity in order to determine the right variety (genotype) for production of safe, effective and quality herbal products.

Traditional methods for plant identification are mainly based on pure morphological traits or time-consuming physiological assays. Now a day, consumers are becoming more cautious about the products; and therefore quality control of the products is becoming very important than never before. It will be better if we use DNA markers, because its use can help us to make sure the quality of the products and to avoid adulteration. Genetic variability testing has been greatly improved by molecular (DNA based) marker techniques; and random amplified polymorphic DNA (RAPD) is one of such technique used to develop molecular markers to distinguish or identify plants. RAPD is the polymerase chain reaction (PCR) based DNA marker, defined as an assay based on the amplification of genomic DNA with single primer of arbitrary nucleotide sequence (Ouborg et al., 1995; Weising et al., 1995; Kumar, 1999; Agarwal et al., 2008). RAPD technique has been widely used for the study of the genetic variability in various higher plants and other living or ganisms (Ouborg et al., 1995; Kumar, 1999; Friesen and Blattner, 2000; Latha et al., 2002; Fu et al., 2003; Nayak et al., 2003; Afzal et al., 2004; Carvalho et al., 2004; Agarwal et al., 2008). The objective of this study was to establish the experimental conditions for *L. pumila* RAPD analyses and to develop DNA (barcode) markers which could help in identification of *L. pumila* and its three varieties from each other.

MATERIALS AND METHOD

Field grown or from nursery, nine accessions were collected randomly from Melaka and Negeri Sembilan states of Malaysia. The collected accessions of *L. pumila* are being maintained in our collection of the medicinal and aromatic plants at Melaka Institute of Biotechnology (Table 1).

Total genomic DNA was isolated from 2 g fresh young leaf tissues of each *L. pumila* accession using method described by Dellaporta et al., (1983) with some minor modifications. The RAPD marker development purpose designed primers with high GC content (60-70 %) were used. All primers used in the study were procured from Proligo and Probes Company.

The RAPD-PCR was performed in a 25 μl PCR reaction volume consisting of 25 ng template DNA, 1X PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTP Mix, 2.5 U of Taq polymerase, and 0.6 μM primer. The amplification was carried out in a programmable thermal cycler. PCR conditions were as follows; hot start at 94°C for 5 min; followed by 50 cycles of 94.0 ℃ for 30 sec, 38.5 ℃ for

Table 1. Accession codes of collected *L. pumila* accessions and their original localities

L. pumila varieties	Collection site (State)	Accession code
L. pumila var. *pumila*	Melaka	K001-a
L. pumila var. *pumila*	Negeri Sembilan	K001-b
L. pumila var. *pumila*	Melaka	K001-c
L. pumila var. *alata*	Melaka	K002-a
L. pumila var. *lanceolata*	Melaka	K003-a
L. pumila var. *lanceolata*	Melaka	K003-b
L. pumila var. *lanceolata*	Melaka	K003-c
L. pumila var. *lanceolata*	Negeri Sembilan	K003-d
L. pumila var. *lanceolata*	Negeri Sembilan	K003-e

1 min, and 74.0 ºC for 1 min. A final step of extension was carried out at 74.0 ºC for 10 min. Ten μl PCR product from PCR of each accession was analyzed by electrophoresis in 1 % agarose-gel with TAE buffer.

Randomly selected RAPD fragments were excised from the agarose-gel and purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Purified RAPD fragments were cloned in pGEM-T-Easy PCR cloning vector (Promega). Recombinant plasmid DNA was isolated using pDNA purification Kit (Promega). The RAPD fragment insert size was confirmed by restriction enzyme analysis; and cloned DNA fragments (RAPD markers) sequencing was carried out from both ends of the DNA strand.

Photograph of stained agarose-gel was used to score the data for RAPD analysis. Comparative analysis of three verities was performed by using two primers selected from 20 RAPD primers. Reproducible and consistent in performance amplified products were chosen for scoring of shared and unshared bands. The presence of a band was scored as 1 and absence was scored as 0, and only fragments (DNA bands) in the range of 200 bp to 2,500 bp were considered in order to increase the data reliability. This data was entered into a binary matrix and a pairwise similarity matrix was constructed using the Jaccard similarity (J) index. The data was quantified by the similarity index, $J_{ij} = C_{ij} / (n_i + n_j)$, where J_{ij} is the number of bands common to individuals i and j, n_i is the number of bands in individual i, and n_j is the number of bands in individual j (Sneath and Sokal, 1973). An Unweighted Pair-Group Method with Arithmetical Averages (UPGMA) cluster based on J values was generated using the NTSYS (Numerical Taxonomy System, Applied Biostatics, Setauket, NY) computer application software (Rohlf, 1998). DNA sequences of the RAPD markers were analyzed online using nucleotide blast (blastn) and blastx programs available at NCBI (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

RAPD comparative analysis was carried out for nine accessions using 20 different random primers to find out the genetic similarities and differences among three varieties of *L. pumila* (Figure 1). Two primers, OPA-1 (5′ CAGGCCCTTC 3′) and OPA-2 (5′ TGCCGAGCTG 3′) (internal code: A10) which gave reliable and consistent RAPD band patterns were selected from 20 primers. The electrophoretic profiles generated by RAPD analysis using OPA-1 random primer is shown in Figure 2.

Analysis of generated RAPD bands was carried out using NTYSYS-pc software that divided nine accessions into two major clusters. Cluster 1 is composed of four accessions namely, K001-a, K003-a, K003-b and K003-c. In this cluster accessions K003-a, K003-b and K003-c appears to be genetically similar, and it suggests that these accessions share the same genetic information.

Cluster 2 is relatively bigger cluster that contains five accessions, namely, K001-b, K001-c, K002-a, K003-d and K003-e with average value J= 0.56. The greatest value of J=0.750 within cluster 2 was observed between accessions K002-a and K001-c. While the smallest value of J=0.200 within this cluster was observed between accessions K003 -e and K001-b.

Genetic relatedness among nine accessions was analyzed by using *Jaccard* similarity coefficient. The dendrogram was constructed based on similarity matrix that had been generated by OPA-1 primer. The similarity matrix obtained using *Jaccard* coefficient is shown in Table 2. The similarity coefficient was in range of 0.167-1.000 in nine accessions of *L. pumila*. The constructed dendrogram to show the genetic relationship between the accessions is shown in Figure 3; and the accession names in respective cluster are stated in Table 3.

To differentiate *L. pumila* var. *alata* and *L. pumila* var *pumila* merely on the basis of morphology (see Figure 1) is relatively difficult due to lack of difference in the leaves and petioles. Results shows that the only one accession of *L. pumila* var. *alata* (K002-a) is closely related to *L. pumila* var *pumila*. This is in accordance with their morphological traits. However, if we have an efficient method to distinguish three varieties of *L. pumila* based on DNA marker will be advantageous and helpful in quality control of the *L. pumila* based products.

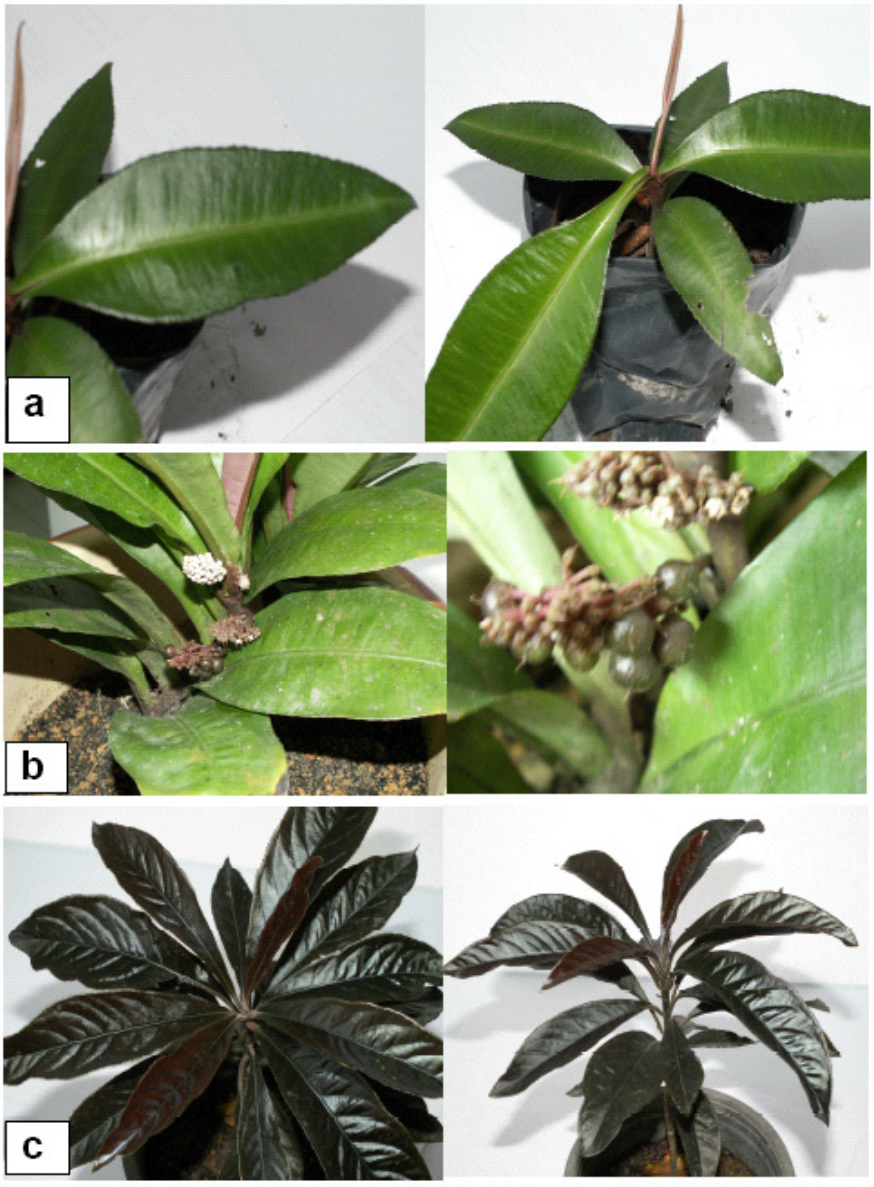

Fig. 1. Three verities of *Labisia pumila* and their observed morphology. (a) *L. pumila* var. *alata*; (b) *L. pumila* var. *pumila*; and (c) *L. pumila* var. *lanceolata*.

The RAPD bands generated by OPA-1 primer can be classified into two groups, polymorphic (variable) and non -polymorphic (constant). Non-polymorphic fragments (DNA bands) are diagnostic for a genus, and operationally identify members of a certain genus exclusively if the respective RAPD fragment is a unique polymorphism in a comparison of genera, genus-specific band or character (Williams et al., 1990). Similarly, RAPD bands non-polymorphic at the species level will operationally identify members of a given species if the fragment is constant among all members of the species, species-specific bands or characters (Welsh and McCelland, 1990). The variety-specific RAPD markers common to all assayed genotypes of respective variety were searched. RAPD technique is known for its usefulness to distinguish varieties precisely (Pradeepkumar et al., 2001). However, in our experiments the RAPD band pattern generated by OPA-1 primer shows polymorphism but variety -specific bands were not observed. The non-polymorphic bands generated by OPA-1 primer can be used for identification of *L. pumila* at specific level although OPA-1

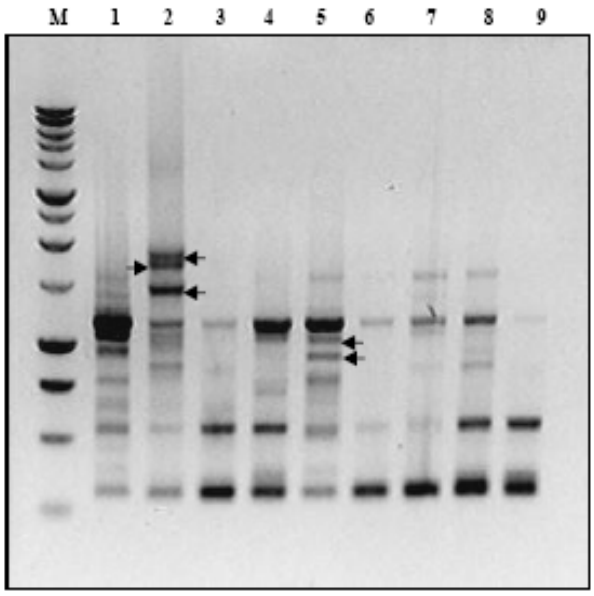

Fig. 2. RAPD amplification products showing polymorphism. Genomic DNA from *L. pumila* accessions was amplified using OPA-1 primer. Arrow indicates positions of some of the prominent genotype-specific polymorphic bands. *Lane M*, 1 kb ladder; *lane* 1-3, RAPD band pattern obtained from 3 accessions of *L. pumila* var. *pumila* (K001-a-c); *lane 4*, RAPD band pattern obtained from one accessions of *L. pumila* var. *alata* (K002-a), and *lane* 5-9, RAPD band pattern obtained from 5 accessions of *L. pumila* var. *lanceolata* (K001-a-e).

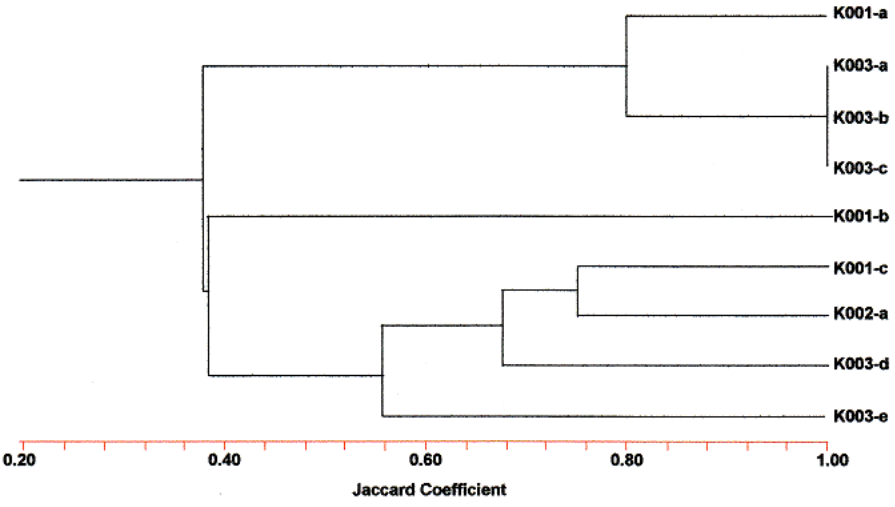

Fig. 3. Cluster analysis of *Labisia pumila* accessions based on RAPD; the accession codes key is given in table 1.

Table 2. Similarity matrix of nine accessions of *L. pumila*

	K001-a	K001-b	K001-c	K002-a	K003-a	K003-b	K003-c	K003-d	K003-e
K001-a	1.0000000								
K001-b	0.5000000	1.0000000							
K001-c	0.3333333	0.4000000	1.0000000						
K002-a	0.5000000	0.3333333	0.7500000	1.0000000					
K003-a	0.8000000	0.3333333	0.4000000	0.6000000	1.0000000				
K003-b	0.8000000	0.3333333	0.4000000	0.6000000	1.0000000	1.0000000			
K003-c	0.8000000	0.3333333	0.4000000	0.6000000	1.0000000	1.0000000	1.0000000		
K003-d	0.5000000	0.6000000	0.7500000	0.6000000	0.3333333	0.3333333	0.3333333	1.0000000	
K003-e	0.1666667	0.2000000	0.6666667	0.5000000	0.2000000	0.2000000	0.2000000	0.5000000	1.0000000

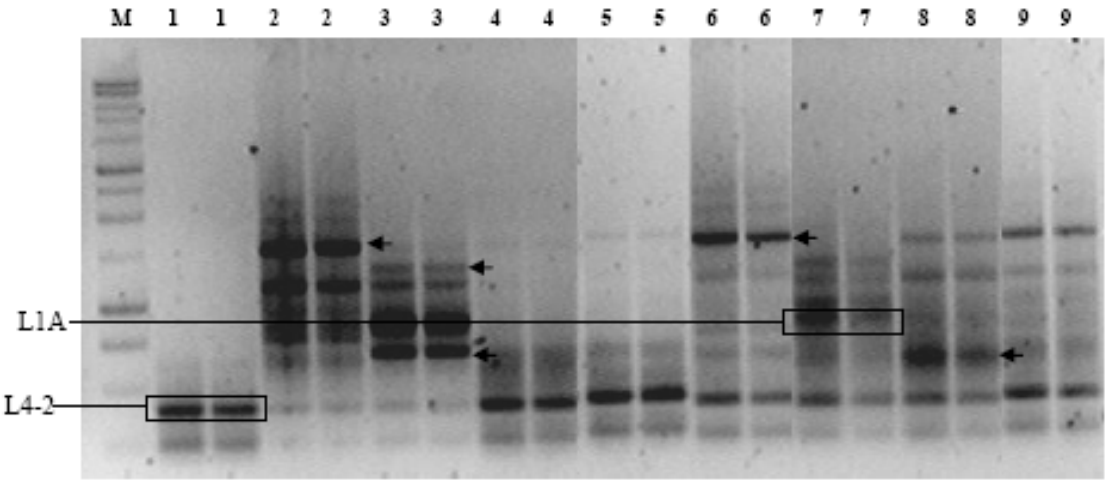

Fig. 4. RAPD amplification products showing polymorphism. Genomic DNA from *L. pumila* accessions was amplified using OPA-2 primer (internal code: A10). Arrow indicates positions of some of the prominent genotype-specific polymorphic bands. *Lane M*, 1 kb ladder; *1-5*, *L. pumila* var. *lanceolata; 6*, *L. pumila* var. *alata*; *7-9*, *Labisia pumila* var. *pumila*. Sequenced RAPD fragments are shown in box, *L1A* and *L4-2* indicates the names of the clones. Note that two lanes were used for gel electrophoresis of PCR products from each accession to have enough DNA for purification and cloning purpose.

primer does not produce any distinctive RAPD band/s which can be used to distinguish three varieties of the *L. pumila*.

The RAPD bands profile generated by OPA-2 (A10) primer also does not show variety-specific RAPD bands which could be used to distinguish three varieties. However, it shows some RAPD bands that are specific to genotypes. Sequencing of the RAPD markers makes them more reliable and could provide useful information to determine trait-specific markers (Venkatachalam et al., 2006). In order to have a DNA sequence based handle, randomly selected two RAPD fragments were cloned, sequenced, analyzed and deposited in gene bank (Table 4). Nucleotide blast of 400 bp RAPD marker sequence showed the 99% homology with a RAPD marker of *Ficus deltoidea* var. *bilobata* (GenBank accession No: EF029047), and 92 % homology with a hypothetical protein of *Gibberella zeae* strain PH-1. Blastx results also showed that 400 bp RAPD marker sequence obtained from *L. pumila* var. pumila contains a sequence that encode for the protein similar to hypothetical protein of *Gibberella zeae* strain PH-1 (Protein accession no: XP_391685). The molecular weight of this 400 bp long RAPD marker is 122579 daltons, and shows 42 % GC content. The RAPD marker sequence (260 bp) which was obtained from *L. pumila* var. *lanceolata* was also analyzed using nucleotide blast and blastx but showed no hits in the database. The molecular weight of this 260 bp

Table 3. Major cluster groups of *L. pumila* accessions based on similarity matrix generated by random primer, OPA-1

Cluster	Accessions
1	*Labisia pumila* var. *lanceolata-a* *Labisia pumila* var. *lanceolata-b* *Labisia pumila* var. *lanceolata-c* *Labisia pumila* var. *pumila-a*
2	*Labisia pumila* var. *pumila-b* *Labisia pumila* var. *pumila-c* *Labisia pumila* var. *alata-a* *Labisia pumila* var. *lanceolata-d* *Labisia pumila* var. *lanceolata-e*

Table 4. RAPD sequence markers of *L. pumila* and their GenBank accession numbers

Clone	*Labisia pumila* variety	RAPD marker length (bp)	GenBank accession number
L1A	*L. pumila* var. *pumila*	400	DQ825505
L4-2	*L. pumila* var. *lanceolata*	260	EF029038

long RAPD marker is 80183 daltons, and shows 38% GC content.

Labisia pumila has been used by many generations of the Malay women in Malaysia; and based on the traditional knowledge of this plant's medicinal value various products such as drinks, herbal tea, extract based capsules and cocktail herbal preparations are coming in the market. However, not much scientific research work has been done on this plant. As of September 13, 2009, in nucleotide database of NCBI there were only 2 nucleotide sequence entries (of two RAPD markers reported in this paper) for *L. pumila* (*L. pothoina* Lindl.). This reflects that *L. pumila* is not widely represented in the nucleotide sequence databases.

CONCLUSIONS

In conclusion, this study provides information about the RAPD profile generated by OPA-1, and OPA-2 (A10) primers. The non-polymorphic bands generated by OPA-1 and OPA-2 (A10) could be used in *L. pumila* identification at species level. Two RAPD marker sequences obtained could also be useful to identify *L. pumila* at species level. The reasonable genetic diversity observed in this study could serve as foundation for the further study on *L. pumila* fingerprinting. However, more accessions (of each variety) should be studied using various RAPD primers to develop more RAPD markers. In addition to this, nuclear ribosomal DNA (rDNA) and mitochondrial DNA polymorphism analysis needs to be carried out and may be helpful to develop more DNA markers to distinguish three varieties of *L. pumila* precisely. Nonetheless, this does not preclude the usefulness of RAPD marker sequences and the reasonable genetic diversity reported in this paper.

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