



## Genetic relationship among *Labisia pumila* (Myrsinaceae) species based on ISSR-PCR

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**ABSTRACT.** The genus *Labisia* (Myrsinaceae) is a popular medicinal plant in Malaysia. We examined the genetic relationship among three varieties of *L. pumila* var. *pumila*, *L. pumila* var. *alata*, *L. pumila* var. *lanceolata* and *Labisia paucifolia* using an ISSR assay. Fifty-eight primers were tested, among which 18 gave reliable polymorphic banding patterns; these yielded 264 polymorphic markers. A similarity matrix was used to construct a dendrogram, and a principal component plot was developed to examine genetic relationships among varieties. Jaccard's similarity coefficient among species ranged from 0.09 to 0.14. At a similarity of 0.117%, species were divided into two main clusters. The mean value of the observed number of alleles, the effective number of alleles, mean Nei's gene diversity, and Shannon's information index were 1.98, 1.64, 0.38, and 0.57, respectively.

**Key words:** Genetic relationship; *Labisia pumila* species; ISSR-PCR

## INTRODUCTION

Myrsinaceae is a widespread family consisting of 30 genera and about 1000 species of tropical plants, of which about 40 species are medicinal in the Asia-Pacific region, particularly for the treatment of inflammation (Shah et al., 2011). Myrsinaceae are valued for their potential medical compounds, quinones and saponins, with a variety of pharmacological activities. The species of Myrsinaceae are widely distributed throughout the tropics and sub-tropics, but are mainly found in South America and South Africa, Asia, with occurrence extending to Australia, New Zealand, and New Guinea (Stone, 1988). *Labisia pumila* is a popular herb in Malaysia, where it is locally known as 'kacip fatimah' and mainly used for the production of herbal products. The increase in knowledge on herbal nutraceuticals and health benefits has driven the growth and demand of herbal products (Karimi et al., 2011a).

Genotype identification and genetic mapping have become increasingly important to the documentation of genetic resources and to the protection of plants. Molecular markers have proven to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations (Guasmi et al., 2012). The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently simple sequence repeats (SSRs) or microsatellites (Gupta and Varshney, 2000; Reddy et al., 2002). The major limitations of these methods are low reproducibility of RAPD, high-cost of AFLP and the need to know the flanking sequences to develop species-specific primers for SSR polymorphism. ISSR-PCR is a technique that overcomes most of these limitations (Zietkiewicz et al., 1994; Taheri et al., 2012). ISSR markers have been efficiently used for the study of the genetic diversity of different medicinal plant species and crops such as *Phaseolus vulgaris* L. (Galván et al., 2003), barley (Yong-Cui et al., 2005), *Artemisia capillaries* (Shafie et al., 2009), *Lippia alba* Mill. (Manica-Cattani et al., 2009), *Achillea millefolium* (Farajpour et al., 2012), *Foeniculum vulgare* Mill (Bahmani et al., 2012), and *Curcuma long* (Singh et al., 2012). No report has yet been published on determination of genetic relationships between *Labisia pumila* species using molecular markers. The objectives of the present study were to determine genetic relationships and identify molecular fingerprints in three varieties of *L. pumila* var. *pumila*, *alata*, *lanceolata* and *Labisia paucifolia* using ISSR markers.

## MATERIAL AND METHODS

### Source of plant materials

Seedlings of *L. pumila* species *Labisia paucifolia* (SK 1910/10), *alata* [Stone 6030 (KLU)], *pumila* [Stone 7233 (KLU)], and *lanceolata* [(Stone 8385 (KLU))] were respectively collected from the following places of origin: Hulu Langat, Selangor; Sungkai, Perak; and Kota Tinggi, Johore. They were raised in a glasshouse at Field 10, University Agriculture Park, Faculty of Agriculture Glasshouse Complex, Universiti Putra Malaysia (101°44'N and 2°58'S, 68 m above sea level) with a mean atmospheric pressure of 1.013 kPa. Healthy and uniform seedlings in terms of leaf numbers were selected for each species.

## DNA extraction

Total genomic DNA was extracted using the modified CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle, 1987) with some modifications. About 0.1 g fresh tender leaf tissue was ground in liquid nitrogen and transferred to a 2-mL microcentrifuge tube. To grind the sample, 800  $\mu$ L extraction buffer containing 2% (w/v) CTAB, 1.4 mM NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% (w/v) PVP and 2% (v/v)  $\beta$ -mercaptoethanol were added and the mixture incubated at 65°C for 1 h. The sample was extracted with 600  $\mu$ L chloroform:isoamyl alcohol [24:1 (v/v)] and the resultant supernatants were transferred to a new microcentrifuge tube. The air-dried pellet was resuspended in 50  $\mu$ L TE buffer 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (pH 8.0) and 2  $\mu$ L (10  $\mu$ g/mL) RNase. The concentration and purity of isolated DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.) and the quality verified by electrophoresis on a 0.8% agarose gel. DNA was diluted with sterile distilled water to a concentration of 50 ng/ $\mu$ L for PCR analysis and kept frozen at -20°C. DNA samples were stored at -20°C prior to ISSR analysis (Taheri, et al. 2012).

## ISSR-PCR analysis

The ISSR was carried out in a 25- $\mu$ L reaction volume including deionized water, 2X DreamTaq™ Green PCR Master Mix (Fermentas, International Inc.), with 2  $\mu$ L 0.6  $\mu$ M oligodeoxynucleotide primer, and 70 ng/ $\mu$ L DNA. All reactions were performed in a thermal cycler (Bio-Rad Laboratories, Inc.) for a total of 40 cycles after an initial denaturation of the template DNA at 94°C for 3 min. This was followed by 10 cycles of 94°C for 40 s, touch-down one-degree decrement for annealing temperature starting at 7°C above  $T_m$  for each primer for 30 s and 72°C for 1 min. This was followed by 30 cycles of 95°C for 40 s, with last annealing temperature for 30 s (Table 1) and 72°C for 1 min, and a final extension of 72°C for 10 min. The amplification products were separated on a 2% agarose gel with a 100-bp DNA ladder (N3231S, Biolabs, Inc.) at 70 V for 90 min. The gel was stained with ethidium bromide, visualized under ultraviolet light and photographed with a gel documentation system (ChemImager™ Gel Doc., Alpha Innotech Corporation, CA, USA).

## Data analysis

The banding pattern for each primer was scored using the 'UVIDOC' software, and fragment sizes were estimated based on a DNA ladder mix (Fermentas International Inc.). Alleles were designated on the basis of fragment size; bands were scored as diallelic (1 = band present, 0 = band absent). To assess the genetic variation between the different varieties of *L. pumila* Benth and *L. paucifolia*, the eighteen ISSR markers were analyzed using NTSYS-pc (Rohlf, 2002). The coefficients of genetic similarity for all pair-wise comparisons were computed using Jaccard's coefficient. A similarity matrix was used to create a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA). The percentages of polymorphic bands (PPB), observed number of alleles ( $N_A$ ), effective number of alleles ( $N_E$ ), gene diversity (h), and Shannon's information index (I) were calculated with the POPGENE software version 1.31 (Yeh et al., 1997) to estimate genetic variation.

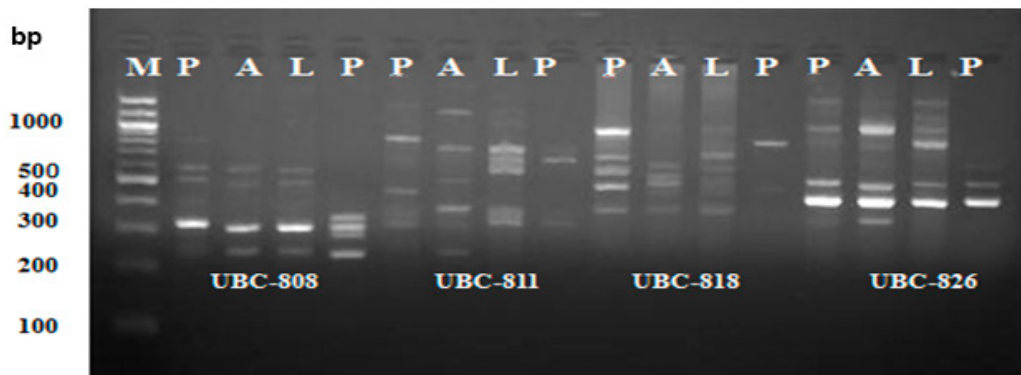
## RESULTS AND DISCUSSION

A total of 58 primers were screened for their ability to amplify genomic DNA in different varieties of *L. pumila* Benth (*alata*, *pumila*, *lanceolata*) and *L. paucifolia* (Figure 1), and they are described in Table 1. Forty of these primers amplified no fragments. The remaining eighteen primers generated a total of 268 fragments with an average of 14.9 polymorphic bands per primer (Figure 1).

**Table 1.** List of ISSR primers and its sequence used for the ISSR analysis.

No.	Primer Code	Sequence (5'-3')	Tm (°C)	Ta (°C)
1	UBC-808	(AG) <sub>8</sub> C	54.6	61-51
2	UBC_811	(GA) <sub>8</sub> C	57.2	64-54
3	UBC_818	(CA) <sub>8</sub> G	57.2	64-54
4	UBC_826	(AC) <sub>8</sub> C	57.2	64-54
5	UBC_834	(AG) <sub>8</sub> YT	56.5	64-54
6	UBC_835	(AG) <sub>8</sub> YC	58.8	66-56
7	UBC_841	(GA) <sub>8</sub> Y	58.8	66-56
8	UBC_842	(GA) <sub>8</sub> YG	58.8	66-56
9	UBC_847	(CA) <sub>8</sub> RC	58.8	66-56
10	UBC_848	(CA) <sub>8</sub> RG	58.8	66-56
11	UBC_850	(GT) <sub>8</sub> YC	58.8	66-56
12	I1	(GA) <sub>9</sub> C	48.5	56-46
13	I2	(GA) <sub>9</sub> T	48.0	55-45
14	I3	(GA) <sub>9</sub> A	49.3	56-46
15	I7	(CT) <sub>9</sub> G	44.9	52-42
16	I74	(ACTG) <sub>4</sub>	54.2	61-51
17	CO9-100	CAGATGGGAGTCAAGTCAAC	60.4	67-57
18	TO5	CGT(TG) <sub>7</sub> T	60.2	67-57

Y = (C, T); R = (A, G). Tm = melting temperature (°C). Ta = annealing temperature (°C).



**Figure 1.** PCR products of genomic DNA from four *Labisia pumila* species with 18 ISSR primers. Lane M = 100-bp DNAMarker; lane P = *pumila*; lane A = *alata*; lane L = *lanceolata*; lane P = *L. paucifolia*.

Of 268 fragments produced, 264 polymorphic bands (98.5%) were used to evaluate genetic relationships between the three varieties of *L. pumila* Benth (*alata*, *pumila*, *lanceolata*) and *L. paucifolia* (Table 2). The percentage of polymorphism in this study was higher than that reported by previous studies using RAPD (63.4%) (Rafii et al., 2012) and ISSR (0.73%)

analysis (Golkar et al., 2011). The number of alleles varied from 5 (I3) to 25 (I2) fragments per primer and ranged from 160 to 2218 bp in size. In this study, the average number of alleles was higher than that reported by Rafii et al. (2012) in RAPD analysis (1.63) and Singh et al. (2012) for ISSR (1.78) analysis in turmeric. All primers tested revealed polymorphisms between the three varieties of *L. pumila* Benth and *L. paucifolia* ranging from 83.3% for primer I3 to 100% for primers UBC-808, UBC-811, UBC-818, UBC-834, UBC-835, UBC-841, UBC-847, UBC-848, UBC-850, I7, I74, C09-100, and TO5. The effective number of alleles per locus ( $N_E$ ) ranged from 1.61 (UBC-850) to 1.76 (UBC-848) with a mean number of 1.64. The difference between the average number of observed alleles and effective number of alleles was due to the uneven frequency of each allele (Babaei et al., 2012). Assuming Hardy-Weinberg equilibrium, the highest mean for Nei's gene diversity (h) was for UBC-841 and UBC-847 (0.40). The average h in the present study was higher than the calculated Nei's gene diversity of *Curcuma long* (h = 0.24) (Singh et al., 2012). Shannon's information index (I) ranged from 0.63 (I2) to 0.58 (UBC-808 and UBC-841) with an average of 0.57, which was higher than that reported in previous studies using ISSR (I = 0.37) (Singh et al., 2012). The overall polymorphism (PPB) for the 18 primers across all three varieties of *L. pumila* Benth and *L. paucifolia* was 98.5%, suggesting that the ISSR procedure constitutes an alternative approach suitable for examining phylogeny at the DNA level. Jaccard's genetic similarity coefficient varied from species to species.

**Table 2.** ISSR primer sequences used for analysis of *Labisia pumila* species with number of bands amplified, number of polymorphic bands, percentage of polymorphic bands (PPB), product size, observed number of alleles (NA), effective number of alleles (NE), Nei's gene diversity (h), and Shannon's information index (I).

Locus	Total No. of bands	No. of polymorphic bands	PPB (%)	Product size (bp)	$N_A$	$N_E$	h	I
UBC-808	11	11	100	847-220	2	1.67	0.39	0.58
UBC_811	16	16	100	1174-224	2	1.62	0.38	0.57
UBC_818	16	16	100	965-362	2	1.65	0.39	0.57
UBC_826	13	12	92.3	1517-300	1.92	1.64	0.31	0.55
UBC_834	19	19	100	1650-260	2.00	1.62	0.38	0.57
UBC_835	16	16	100	1834-226	2.00	1.60	0.37	0.56
UBC_841	15	15	100	1543-264	2.00	1.65	0.40	0.58
UBC_842	17	16	94.1	790-160	1.94	1.62	0.38	0.55
UBC_847	16	16	100	1543-380	2.00	1.64	0.40	0.57
UBC_848	16	16	100	987-246	2.00	1.76	0.38	0.57
UBC_850	19	19	100	1913-322	2.00	1.61	0.38	0.57
I1	11	10	90.9	1400-450	1.90	1.63	0.36	0.53
I2	25	25	100	1277-407	2.00	1.63	0.38	0.56
I3	6	5	83.3	1200-530	1.80	1.63	0.38	0.56
I7	6	6	100	1537-650	2.00	1.63	0.38	0.56
I74	12	12	100	2218-240	2.00	1.63	0.38	0.56
CO9-100	16	16	100	1853-222	2.00	1.63	0.38	0.56
TO5	18	18	100	1017-370	2.00	1.63	0.38	0.56
Total	268	264						
Mean	14.9	14.6	98.5		1.98 ± 0.12	1.64 ± 0.15	0.38 ± 0.06	0.57 ± 0.08

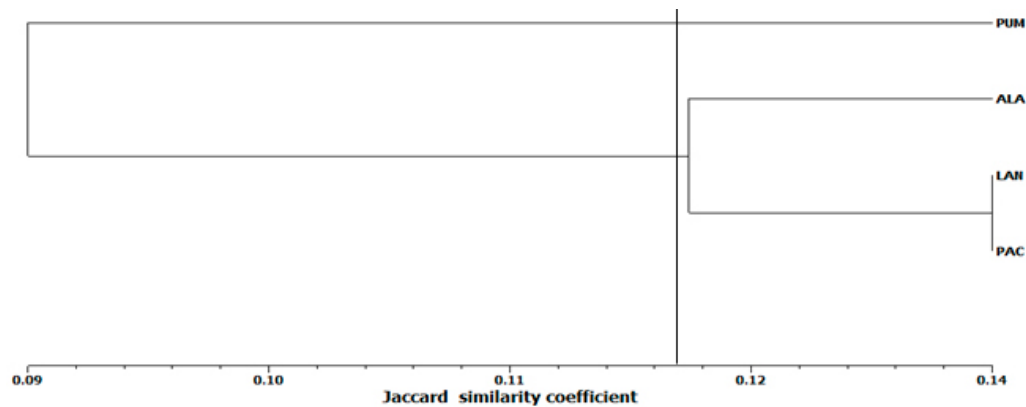
The similarity matrix (Table 3) indicated that the lowest genetic similarity (0.06) was between *L. pumila* var. *pumila* and *L. pumila* var. *alata*, and the highest similarity (0.14) was between *L. pumila* var. *lanceolata* and *L. paucifolia*. A comparable wide range in similarity values had also been observed between cyclamen accessions (Naderi et al., 2009).

UPGMA cluster analysis of Jaccard's similarity coefficient was employed to observe

the genetic relationship between the three varieties of *L. pumila* Benth and *L. paucifolia*. According to the dendrogram constructed using ISSR markers, at the similarity level of 0.117, the species were divided into two main clusters (Figure 2). In group I, *L. pumila* var *pumila* was separated from the other species with only 0.09 similarity. On the other hand, *L. paucifolia*, *L. pumila* var. *lanceolata*, and *L. pumila* var. *alata* were assigned to group II. *L. paucifolia* and *L. pumila* var. *lanceolata* showed the most similarity (0.14) and formed the first subcluster in group II. The lone species *L. pumila* var. *alata* showed a JSI = 0.118 with *L. paucifolia* and *L. pumila* var. *lanceolata*, and formed the second subcluster in group II. Different hierarchical positions in the dendrogram of the three varieties of *L. pumila* Benth and *L. paucifolia* studied showed that genomes of each variety are not exactly the same. It is imperative to differentiate between *L. pumila* (Myrsinaceae) species in terms of their physical and chemical characteristics as well as biological activity. For instance, the shape of the petiole base in *L. pumila* var. *pumila* and *L. pumila* var. *alata* is marginate and winged, respectively, but long in *L. pumila* var. *lanceolata* and *L. paucifolia* (Stone, 1988). Additionally, a previous study on chemical profiling demonstrated that *L. pumila* var. *pumila* had higher total flavonoids compared to *L. pumila* var. *alata*, *L. pumila* var. *lanceolata*, and *L. paucifolia*., while *L. pumila* var. *alata* contained higher total phenolics and antioxidant activities compared to the other species mentioned (Karimi et al., 2011b).

**Table 3.** Genetic similarity indices between each pair of the four *Labisia pumila* species based on ISSR analysis.

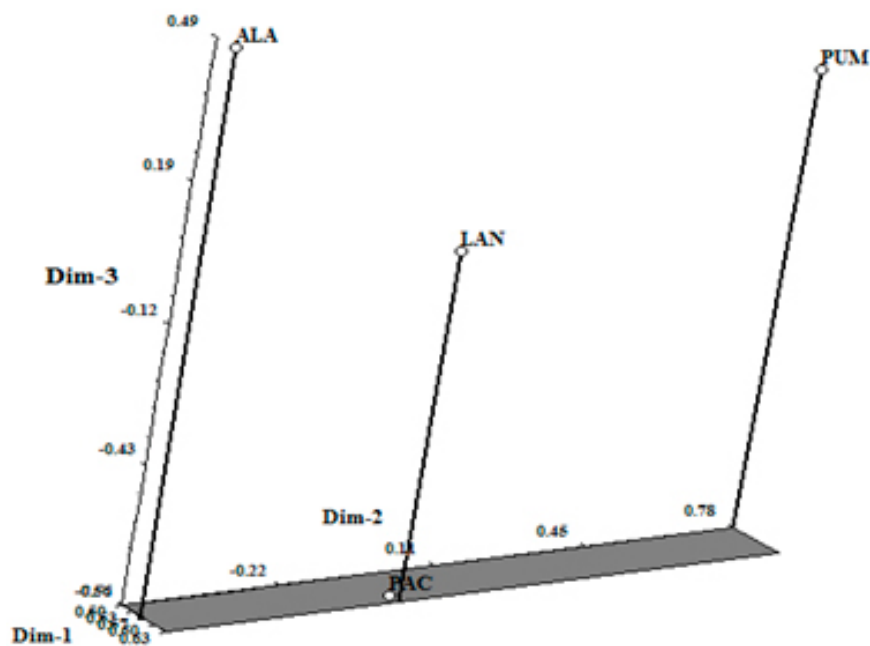
Species	<i>L. pumila</i>	<i>L. alata</i>	<i>L. lanceolata</i>	<i>L. paucifolia</i>
<i>L. pumila</i>	1.00			
<i>L. alata</i>	0.0639535	1.00		
<i>L. lanceolata</i>	0.1079545	0.1288344	1.00	
<i>L. paucifolia</i>	0.0972222	0.1136364	0.1357143	1.00



**Figure 2.** Dendrogram demonstrating the relationships among the four *Labisia pumila* species based on ISSRs.

Group constellations were also independently developed by using principal component analysis (PCA) to verify the grouping obtained through the dendrogram. PCA further helped in depicting the variation between the three varieties of *L. pumila* Benth and *L. pauci-*

*folia* in a three-dimensional mode. The first two PCA axes of the mentioned species are shown in Figure 3. PCA divided the *Labisia* species into two groups, a result similar to the dendrogram derived by UPGMA. Three principal components with eigenvalues greater than unity extracted a cumulative of 78.5% of the variance in three varieties of *L. pumila* Benth and *L. paucifolia*. The three components, PC1, PC2, and PC3 showed 33.1, 23.4, and 21.9% variation, respectively. PCA showed the three-dimensional relationships that describe portions of the genetic variance in a dataset for three varieties *L. pumila* Benth and *L. paucifolia*.



**Figure 3.** Principal component analysis map for ISSR markers of the four *Labisia pumila* species.

Although there are published reports on the use of ISSR molecular marker techniques to analyze various species, there is no report on the use of molecular markers in *L. pumila* Benth. There is a significant need to develop a molecular marker system to address the many unresolved questions related to the genetic history, status, and trends for *L. pumila* species. For major crop plants, the many advantages of microsatellites make them the preferred marker for genetic studies (Agarwal et al., 2008; Kalia et al., 2011). However, a PCR-derived marker, such as ISSR, that uses non-specific primers, is the most suitable for most wild plant species, such as *L. pumila* species, due to the lack of locus-specific genetic sequence information (Nybom and Bartish, 2000). Thus, ISSR markers were utilized because they are relatively inexpensive, have modest equipment requirements, and are reliable when good scientific practices are rigorously applied (Skroch and Nienhuis, 1995). On the basis of our results, we believe that an ISSR marker system could be used extensively as a practical and reliable genetic tool for the strategic genetic conservation of *L. pumila* species.

## CONCLUSION

This is the first attempt to use molecular markers to investigate genetic relationships of *L. pumila* species, and the information generated herein may be useful for the improvement of this crop and could guarantee a promising future for breeding programs. Further studies examining SSR loci could explain more completely the diversity between these species.

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