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Morphological and Genetic (RAPDs) Characterization of *Munronia pinnata* (Meliaceae)

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ABSTRACT. *Munronia pinnata (Wall) Theob. (Binkohomba) of the family Meliaceae is a medicinal shrub which grows naturally as an understorey species in the dry, dry-mixed to wet evergreen forests of Sri Lanka. This is considered a threatened species. The entire plant has been used for medicinal purposes and the national demand is provided mainly by collection from natural populations resulting in over-exploitation of the species. Although the characterization of this plant is a fundamental requirement in the conservation and utilization of it, little is known about its morphological and genetic characterization. Consequently, morphological and genetic variations of five populations of M. pinnata were investigated using eight morphological characters and 219 randomly amplified polymorphic DNA (RAPD) products.*

Based on these eight morphological characters, M. pinnata plants collected from five regions were mainly grouped into four clusters. Variation in morphological characters was more frequently observed between populations than within populations. Five (Ritigala) and seven (Wellawaya) leaflet populations separately clustered, whilst three leaflet populations formed two distinct clusters. Dendogram obtained from RAPD markers revealed that the five populations could be grouped into three main clusters, namely, seven (Wellawaya), five (Ritigala) and three leaflet (Haldummulla, Sinharaja and Naula) populations, suggesting that the morphological variation of three leaflet population may be due to environmental variability. Findings of the present study about population distribution and variation of M. pinnata are useful for designing future coservation and utilization strategies of this plant.

INTRODUCTION

Munronia pinnata (Wall) Theob. (Binkohomba) of the family Meliaceae is a small, hardy, perennial medicinal shrub in Sri Lanka (Jayaweera, 1982; Bandaranaike and Sultanbawa, 1991; Dassanayake *et al.*, 1995). The species grows naturally as an understorey plant in the dry, dry-mixed to wet evergreen forests. Presence of this plant has been recorded in Ritigala, Doluwa, Naula, Maturata, Haldummulla, Wellawaya, Balangoda, Kataragama, Sinharaja, Pollonnaruwa, Sigiriya, Lunugala and Botale areas (Jayaweera, 1982; Dassanayake *et al.*, 1995). It is included in the list of the most important 50 medicinal plant species in the country (SLSUMPP, 1999).

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The entire plant has been used for medicinal purposes to treat fever, dysentery, leprosy and other skin diseases and purification of blood (Jayaweera, 1982). The annual requirement of *M. pinnata* in the country is about 10,000 kg of dried material, which requires almost 30,000 plants (Sumithraarachi, 1996). The farmgate price of dried *M. pinnata* is very high and ranges from Rs. 2,000-3,500 per kg. Since it has not been cultivated as a crop, the national requirement is provided mainly by collection from natural populations. The high demand and high price of dried material of *M. pinnata* have resulted in over-exploitation of the species. Further, ever increasing deforestation and animal browsing have also resulted in reduction of populations. Therefore, conservation and sustainable utilization of *M. pinnata* has become an urgent national requirement.

It is well known that characterization of a plant is a fundamental requirement in its conservation and utilization. This is important for *M. pinnata*, since the species has shown morphological differentiation based on the leaflet number per leaf (Marasinghe, 1991). Reliable information on the distribution of the genetic variation within and between geographical regions is important to establish the distribution network of plant populations. Identification of the variation by morphological, biochemical and genetic characterization may provide valuable information with respect to its taxonomy, phylogenetic relatedness, and genetic and environment interactions. However, little is known about the morphological and genetic variation of *M. pinnata* populations in Sri Lanka.

Morphological characterization will be useful in separation of the population into different types and provenances. However, some morphological characters are environmentally dependent and some physiological characters are not reflected by morphology (Weising *et al.*, 1995). Thus, application of molecular markers is an useful alternative to avoid the drawbacks in morphological characterization. Various molecular tools have been developed to identify the population structure of tree species (Gillies *et al.*, 1997). DNA based methods, particularly those using PCR are becoming increasingly popular in diversity studies, as they can be conducted on small amount of plant material. Among PCR based techniques, Randomly Amplified Polymorphic DNA (RAPD) has proven to be a powerful tool over the conventional approach of morphological characterization in the assessment of genetic variation of plant populations (Welsh and McClelland, 1990; Williams *et al.*, 1990). RAPD has been successfully used to elucidate genetic relationships among accessions of rice, rubber, tea and sugarcane in Sri Lanka (Ubayasena and Perera, 1999). The present study is an attempt to investigate the morphological and genetic variation of *M. pinnata* populations in Sri Lanka.

MATERIALS AND METHODS

Plant material

M. pinnata plants were collected from Naula, Ritigala, Haldummulla, Wellawaya and Sinharaja to represent variation in leaflet number per leaf and agroecological regions. About 5-10 plants were collected from each location to minimize disturbances to the natural populations since the species is categorised as

threatened. Collected plants were potted, labelled and placed in a green house at the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya.

Morphological characterization

Morphological characterization of *M. pinnata* was done using eight morphological characters as shown in Table 1. The variation of leaflet number in *M. pinnata* is shown in Plate 1. Each plant maintained at the green house was assessed for the selected morphological characters using a scoring system (Table 1). Data was analyzed using factor and principle component analysis of SAS, which has been used to characterize germplasm information (Piyasiri *et al.*, 2001).

Table 1. Eight morphological characters identified for *Munronia pinnata*.

Morphological character	Scoring system
1. Leaf habit	1=erect, 2=intermediate and 3=drooping
2. Leaflet number	1=three leaflet, 2=five leaflet, 3=seven leaflet, 4=nine leaflet, 5=eleven leaflet
3. Leaf shape	1=round, 2=oblong
4. Leaf margin	1=entire, 2=undulate, 3=serrated
5. Colour of middle vein	1=silver, 2=white, 3=not clear
6. Hairiness of plant	1=maximum hairs, 2=less hairs, 3=no hairs
7. Colour of flower	1=white, 2=purple
8. Length of stigma	mm

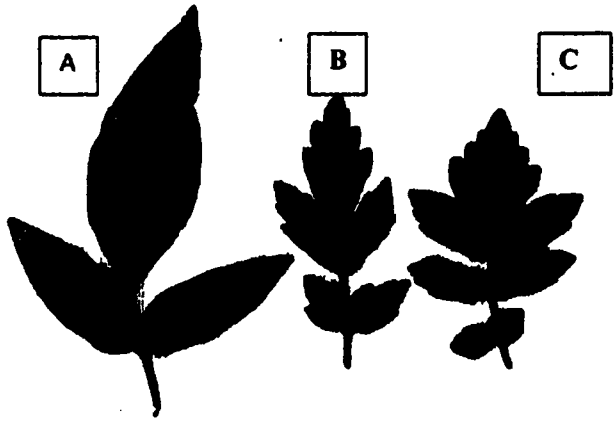

Plate 1. Variation of leaflet number in *Munronia pinnata*.
[Note: A, B and C represent plants with 3 (Naula), 5 (Ritigala) and 7 (Haldummulla) leaflet types, respectively].

Molecular characterization

Sampling of plants for DNA extraction and visualization

Preliminary observations revealed that at least 3 g of tender leaf material was required for effective DNA extraction. Because a single plant could not provide 3 g of tender leaf material, samples were collected and bulked from at least two plants from each location as shown in Table 2. DNA was extracted three times according to the growth rate of the plants.

Table 2. Sampling of plant material for DNA extraction.

Name	First extraction	Second extraction	Third extraction
Ritigala	1, 3	2, 5	1, 5
Haldummulla	1, 4, 5	3, 4, 5	1, 4, 6
Wallawaya	1, 2	3, 6	4, 5
Naula	1, 2	3, 4	1, 4
Sinharaja	1,2	-	-

Individual plants collected from each location was labeled from 1, 2.....n.
Only combinations shown in the Table are used for extraction of DNA from each location.

DNA was extracted following the modified CTAB protocol described by Weising *et al.* (1995). Tender leaf tissues were ground in a cold mortar in liquid nitrogen to form a fine powder. The powder was collected into a polyprophylene tube and incubated at 60°C for 30 min after adding 15 ml of pre-warmed CTAB buffer. 30 µl of β-mercaptoethaonol was added to the 4% CTAB buffer just before using. Then, chloroform:isoamyl alcohol mixture in the ratio of 24:1 was added to the sample and kept for another 10 min. The extracts were collected into glass tubes after centrifugation at 5,000 rpm for 10 min. This step was repeated 2–3 times until a clear solution appeared. The DNA complex was precipitated with ice-cold isopropanol. The DNA pellet was washed with 10 mM ammonium acetate and 70% ethanol solution and allowed to dry and resuspended in 500 µl of TE buffer. DNA concentration was estimated using a spectrophotometer. Total DNA was diluted with sterile distilled water to a concentration of 30 ng/µl.

PCR amplification and agarose gel electrophoresis

The 20 µl reaction mixture consisted of the following ingredients: 30 ng template DNA; 200 µm each dATP, dCTP, dGTP, dTTP; 10 pmoles of primer; 1 unit of *Taq* polymerase; 10x incubation mix and $MgCl_2$. Thirteen decamer oligonucleotides (Operon Technologies) primers, namely OPA 20, OPC 4, OPC 15, OPD 1, OPD 7, OPD 8, OPD 12, OPK 13, OPK 16, OPM 5, OPM 6, OPM 13 and OPM 15 were used for PCR amplification. Amplification was performed in Perkin Elmer PCR machine (Gene

Amp PCR system 9600) using the following programme: initial 3 min denaturation at 94°C followed by 40 cycles of 1min at 93°C, 3 min at 35°C and 2 min at 72°C. The products were stored at 4°C.

All PCR products were separated by electrophoresis in 1.5% agarose gel in 1xTAE buffer and stained with ethidium bromide (5 µg/ml) and viewed under ultraviolet light. The reactions were performed at least two times to confirm the reproducibility of the amplified DNA fragments with each primer.

Data scoring and analysis

Data generated with the 13 primers were used to compile a binary data matrix for cluster analysis for the presence (1) or absence (0) of each scorable and reproducible band. The data was analysed using SAS.

RESULTS AND DISCUSSION

Morphological characterization

Large variability was observed in the plant population with respect to the eight morphological characters considered in the study. Plate 1 shows variation of leaflet number in *M. pinnata*. Results of the principal component analysis showed that the first two principal components obtained for eight morphological characters explained 81% of the accumulated variation of *M. pinnata* germplasm (Table 3), which is adequate to explain total variability of populations. In factor analysis a three factor model explained 87% of total variation (Table 4). The first factor explained 44% of the variation and was associated with leaf habit. The second factor was associated with margin of leaf, colour of middle vein and length of the stigma and explained 25% of the variation whilst the third factor explained 18% of total variation and was associated with leaf number, leaf shape and hairiness of the plant (Table 4).

Table 3. Eigen values of correlation matrix of principal components.

	Eigen value	Proportion	Cumulative
Prin 1	3.76	0.54	0.53
Prin 2	1.91	0.27	0.81

In the dendrogram obtained from morphological characters, five populations were grouped to form four major clusters at the distance of 0.7 (Fig. 1). Three leaflet plants were grouped into two distinct clusters, one with Sinharaja and Haldummulla populations and the other with Naula population. Wellawaya (seven leaflets) and Ritigala (five leaflets) populations were separately grouped into two clusters. Morphological characters in the three leaflet plants collected from different regions

showed variation among them. Therefore, Naula population, which possessed three leaflets, and has other characters such as silver middle vein and entire leaf margin, it was grouped into a separate cluster. These results suggest that morphological variation of *M. pinnata* is mainly between populations. Minimum morphological variation in all characters was observed within a given population.

Table 4. Rotated factor loading, Eigen values, percentage of total variance obtained for three factor model of seven characters.

Character	Rotated factor loading		
	Factor 1	Factor 2	Factor 3
1. Leaf habit	**0.7792**	0.5459	-0.4059
2. Leaflet number	0.0097	0.3500	**0.9056**
3. Leaf shape	0.2871	-0.1036	**0.9161**
4. Plant hairiness	0.2708	-0.2972	**-0.7909**
5. Leaf margin	-0.2018	**0.8137**	-0.0251
6. Middle vein	0.0815	**0.7535**	0.5405
7. Length of stigma	-0.0614	**0.9298**	-0.2438
Eigen values	3.5170	1.9918	1.4724
Population variation Explained (%)	44	25	18
Cumulative population Variance explained (%)	44	69	87

No variation was observed for the colour of flower and was thus excluded from the analysis.

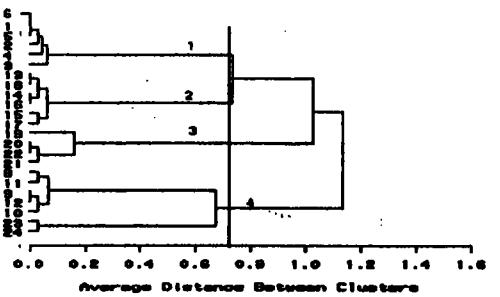

Fig. 1. Dendrogram of morphological characters of *Munronia pinnata* individuals from five populations.
[Note: Clusters 1 and 2 consists of populations from Wellawaya and Ritigala. Cluster 3 consists of population from Naula. Cluster 4 consists of populations from Haldummulla and Sinharaja].

Molecular characterization

Thirteen random primers used in the study generated a total of 278 scoreable bands (amplified products) of which 219 were polymorphic (Table 5; Plates 2, 3 and 4)

and 59 were monomorphic. The approximate size of amplified products ranged from 100 bp to 2 kbp (Plates 2, 3 and 4). Based on the amplified products, the five populations could be grouped to form three major clusters at the average genetic distance of 0.87 (Fig. 2). Cluster 1 consists of seven leaflet population from Wellawaya, while cluster 2 consists of three leaflet populations collected from Haldummulla, Naula and Sinharaja. The third cluster comprised only five leaflet plants from Ritigala.

Table 5. Primer sequence and number of polymorphic bands for each primer.

Primer	Primer sequence	No of polymorphic bands
A20	GTTGCGATCG	21
C4	CCGCATCTAC	33
C15	GACGGATCAG	20
D1	ACCGCGAAGG	14
D7	TTGGCACGGG	22
D8	GTGTGCCCCA	19
D12	CACCGTATCC	20
K13	GGTTGTACCC	16
K16	GAGCGTCGAA	16
M5	GGGAACGTGT	6
M6	CTGGGCAACT	6
M13	GGTGGTCAAG	18
M15	GACCTACCAC	8
Total		219

It was clear from the results that the population of *M.pinnata* grouped into four clusters based on the morphological features, because three leaflet populations formed two groups indicating environmental modification of morphological features. However, molecular (genetic) information produced only three clusters. It can be suggested that three, five and seven leaflet forms are genetically sub structured indicating that three leaflet groups although collected from different regions, are genetically closely related. Accordingly, for conservation purposes, it is important to demarcate populations of three, five and seven leaflet types from various areas.

This is the first record of the morphological and molecular characterization of *M. pinnata* in Sri Lanka. Although the occurrence of variation of leaflets in *M. pinnata* has also been observed by Marasinghe (1991), this is the first instance that showed morphological differences are correlated to environmental differences and genetic variation (Figs. 1 and 2). The other members of the family Meliaceae also possess similar phenomenon in leaflet numbers, (personal communication, Meliaceae internet discussion group) however, very little is known about their association with respect to geographical variation.

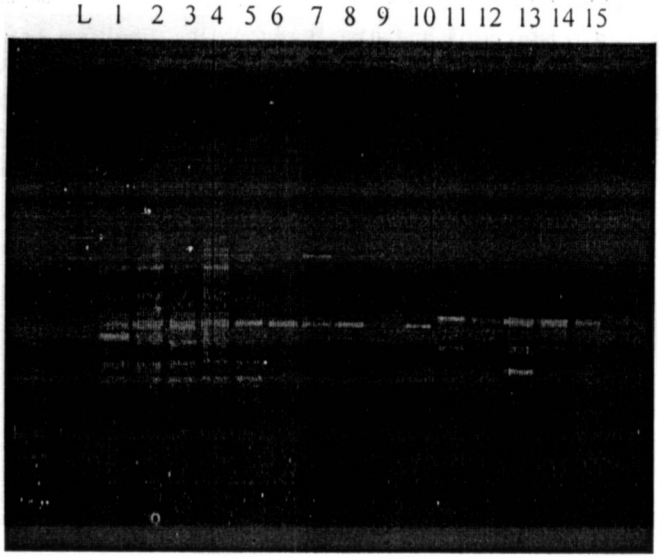

Plate 2. **RAPD amplification products of *Munronia pinnata* populations.**

[Note: Lane L represents 1 kb DNA ladder, Lanes 1-5, 6-10, 11-15 represent amplified products obtained from OPA20, OPC14 and OPD1 primers, respectively. In each primer, five lanes represent DNA extracted from Wellawaya, Haldummulla, Naula, Sinharaja and Ritigala, respectively].

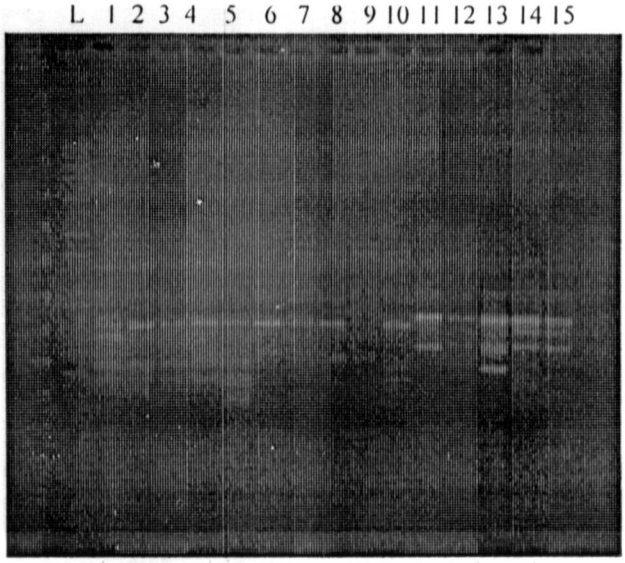

Plate 3. **RAPD amplification products of *Munronia pinnata* populations.**

[Note: Lane L represents 1 kb DNA ladder, Lanes 1-5, 6-10, 11-15 represent amplified products obtained from OPC15, OPK13 and OPK16 primers, respectively. In each primer, five lanes represent DNA extracted from Wellawaya, Haldummulla, Naula, Sinharaja and Ritigala, respectively].

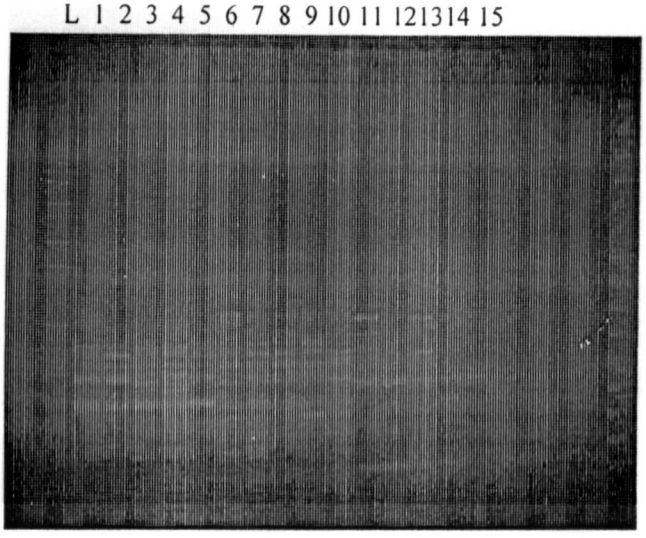

Plate 4. **RAPD amplification products of *Munronia pinnata* populations.**
[Note: Lane L represents 1 kb DNA ladder, Lanes 1-5, 6-10, 11-15 represent amplified products obtained from OPD7, OPD8 and OPD12 primers, respectively. In each primer, five lanes represent DNA extracted from Wellawaya, Haldummulla, Naula, Sinharaja and Ritigala, respectively].

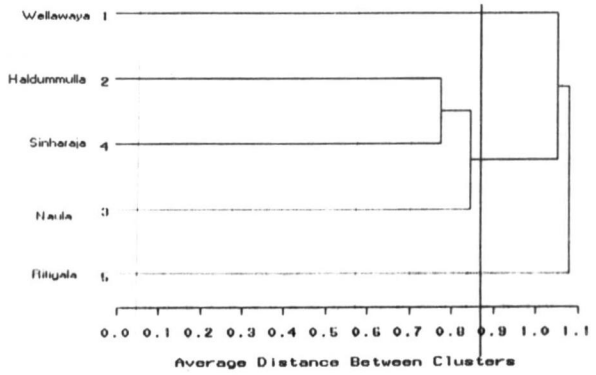

Fig. 2. Genetic relatedness among five populations showing three distinct clusters of *Munronia pinnata* based on 278 RAPD bands from 13 primers.

Studies in genetic variation of *Cedrela odorata* in family Meliaceae (Gilles *et al.*, 1997) suggested that RAPD was more appropriate for analysis of genetic variability in closely related genotypes. In addition, the ability of RAPD markers to discriminate between populations may be particularly helpful in designing effective conservation strategies that reflects the true picture of the variation present within the species (Dawson *et al.*, 1993).

Further, molecular analysis is needed with larger sample size to confirm the genetic relationship of *M. pinnata*. Morphological and genetic characterization information and, correlation analysis with chemical characterization are essential for identification of individuals for multiplication and also for demarcation of effective populations for conservation and utilization of this species. Understanding of the reproductive biology of the species will resolve the reasons for morphological and genetic variations of *M. pinnata* populations.

CONCLUSIONS

The results of this investigation indicated that there is genetic and morphological differentiation of *M. pinnata* among populations sampled from Haldummulla, Sinharaja, Naula, Ritigala and Wellawaya regions in Sri Lanka. The existence of such pronounced population differentiation has profound implications for the conservation and sustainable use of the species.

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