CHAPTER IV

Amplified Fragment Length Polymorphisms (AFLPs)

AFLP technique has been identified as a robust DNA fingerprinting technique (Vos *et al.*, 1995) that detects significant levels of polymorphism between accessions. AFLP markers are highly repeatable (Breyne *et al.*, 1997), provide broad genomic coverage and a virtually limitless number of genetic markers. AFLP can be utilized not only to determine the relationship of closely related species, but as a tool to authenticate material in herbal remedies through the use of genetic fingerprinting. The present work utilized the primers combinations. The polymorphic bands were generated. Then, AFLP amplification bands showed a species-specific band of *L. speciosa* for authentication of plant materials. In addition, AFLP binary data was used for studying the genetic relationship in *Lagerstroemia.*

4.1 Plant materials

The leaves of four *Lagerstroemia species*, *L. speciosa*, *L. macrocarpa*, *L .loudonii* and *L. floribunda* were used in this study. A closely related species, *Lawsonia inermis*, was used as an outgroup. All of the collected plant materials and their localities are listed in **Table 4.1**. The genuine leaves specimens were authenticated by Associate Professor Thatree Phadungchareon. The specimens have been deposited in the department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Table 4.1 Plant materials for AFLPs evaluation.

Plant species	No.	Voucher no.	Locations
L. speciosa	LS1	LS1WT200908	Rajamangala University of Technology Phra Nakhon. Bangkok
	LS2	LS2WT220908	The Faculty of Pharmaceutical Sciences, Chulalongkorn university
	LS3	LS3WT070109	The Faculty of Veterinary Sciences. Chulalongkorn university
	LS4	LS4WT150109	The Royal Turf Club of Thailand Under Royal Patronage, Bangkok
	LS5	LS5WT130309	The Central Botanical Garden (Pukae), Saraburi Province
	LS6	LS6WT270309	The Intanin field, Maejo University, Chiang Mai Province
	LS7	LS7WT270309	The Maharaj Nakorn Chiang Mai Hospital, Chiang Mai Province
	LS8	LS8WT140409	Wat Pa-ngun, Sawee District, Chumporn Province
L. macrocarpa	LM1	LM1WT051008	The Faculty of Political Sciences, Chulalongkorn university
	LM2	LM2TP261208	Blood Bank Section, Faculty of Medicines, Chulalongkorn university
	LM3	LM3WT270329	The Intanin field, Maejo University, Chiang Mai Province
	LM4	LM4WT270309	The Maharaj Nakorn Chiang Mai Hospital, Chiang Mai Province
L. loudonii	LL1	LL1WT130309	The Central Botanical Garden (Pukae), Saraburi Province
	LL2	LL2WT190409	Rajavinit Mathayom school, Bangkok
L. floribunda	LF1	LF1WT130309	The Central Botanical Garden (Pukae), Saraburi Province
	LF2	LF2WT190409	The Royal Turf Club of Thailand Under Royal Patronage, Bangkok
Lawsonia inermis	LAW	LAWSW180809	Jatujak market, Bangkok

4.2 Methods

4.2.1 Genomic DNA extraction

Approximately 3 g of leaves was ground in liquid nitrogen with mortar and pestle. Isolation of total genomic DNA followed the protocol described by Doyle and Doyle (1990), which is a CTAB-based extraction protocol. The concentration of genomic DNA was estimated by using spectrophotometric measurement of UV absorbance at 260 nm.

4.2.2 AFLPs procedure

The AFLPs procedure was carried out as reported by Vos *et al.* (1995) with a few modifications.

4.2.2.1 Digestion of genomic DNA

Approximately 100 ng/µl of genomic DNA was digested with two restriction enzymes, *EcoR*I HF and *Mse*I in 10x buffer 4. The reaction was incubated at 37°C for 2 hours. The complete digestion products were investigated by 1% agarose gel electrophoresis in 0.5x TBE using 10 µl of digestion product.

Table 4.2 Reaction mixture for digesting genomic DNA with restriction enzymes.

Digestion components	Volumes (µl)
100 ng/µl Genomic DNA	5.00
10 unit/µl *EcoR*I (NEB, United Kingdom)	0.50
10 unit/µl *Mse*I (NEB, United Kingdom)	0.50
10X buffer 4 (NEB, United Kingdom)	4.00
ddH_2O	30.00
Total	**40.00**

4.2.2.2 Ligation of genomic DNA

The digestion genomic DNA fragment was ligated to *EcoR*I-adapter and *Mse*I-adapter (**Table 4.3**) by adding 10 µl of ligation master mix. The reaction was incubated at 37°C for at least 3 hours (preferably overnight). The completeness of ligation process was detected by 1% agarose gel electrophoresis in 0.5x TBE using 5 µl of ligation reaction and 1 µl of 6x loading dye. Each ligation reaction was diluted as ten-folded with ddH_2O. The aliquots were stored at -20°C

Table 4.3 Sequences of adapters and primers used for AFLPs analysis.

Name/Abbreviation	Type	Sequence (5'--- 3')
*EcoR*I-adapter		CTC GTA GAC TGC GTA CC AAT TGG TAC GCA GTC TAC
MseI-adapter		GAC GAT GAG TCC TGA G TAC TCA GGA CTC AT
ER1A	Primer +1	AGA CTG CGT ACC AAT TCA
ER3ACC	Primer +3	AGA CTG CGT ACC AAT TCA CC
ER3AAC	Primer +3	AGA CTG CGT ACC AAT TCA AC
ER3AAG	Primer +3	AGA CTG CGT ACC AAT TCA AG
MS3C	Primer +1	GAT GAG TCC TGA GTA AC
MS3CTA	Primer +3	GAT GAG TCC TGA GTA ACT A
MS3CAG	Primer +3	GAT GAG TCC TGA GTA ACA G
MS3CAT	Primer +3	GAT GAG TCC TGA GTA ACA T
MS3CTT	Primer +3	GAT GAG TCC TGA GTA ACT T
MS3CTG	Primer +3	GAT GAG TCC TGA GTA ACT G
MS3CAC	Primer +3	GAT GAG TCC TGA GTA ACA C

Table 4.4 Reaction mixture for nucleotide adapter ligation.

Ligation components	Volume (µl)
5 µM EcoRI-adapter (Eurofins MWG Operon, Germany)	1.00
50 µM MseI-adapter (Eurofins MWG Operon, Germany)	1.00
T4 DNA Ligase (NEB, United Kingdom)	0.40
10x ligation buffer (NEB, United Kingdom)	1.00
ddH_2O	6.60
Total	10.00

4.2.2.3 Pre-selective amplification

Five microliters of diluted ligation product were amplified using ER1A and MS1C primers (**Table 4.3**). Each reaction was composed of 50 µl pre-amplification primer mixture (**Table 4.5**). PCR was performed in a C1000 Themal Cycler (Biorad, USA) and used the following pre-selective PCR program. The pre-amplification PCR product was diluted to ten-folded with ddH_2O, mixed and placed in -20°C or proceeds to next step.

Table 4.5 Reaction mixture for pre-amplification reaction.

Ligation components	Volume (µl)
Digestion – ligation product	5.00
10 µM ER1A (Eurofins MWG Operon, Germany)	1.00
10 µM MS1C (Eurofins MWG Operon, Germany)	1.00
10x PCR buffer (Invitrogen, USA)	5.00
50 mM $MgCl_2$ (Invitrogen, USA)	1.50
10 mM dNTPs (Fermentas, Canada)	1.00
5 U/µl Taq polymerase (Invitrogen, USA)	0.50
ddH_2O	35.00
Total	50.00

Pre-amplification PCR program (C1000 Thermal Cycler, Biorad)

Step 1:	Predenaturation step	95°C	3 min.
Step 2:	Denaturation step	95°C	30 sec.
Step 3:	Annealing step	56°C	1 min
Step 4:	Extension step	72°C	1 min
Step 5:	20 cycles; step 2 to step 4		
Step 6:	Final extension step	72°C	10 min
Step 7:	Hold	4°C	∞
Step 8:	End		

4.2.2.4 Selective amplification

Three microliters of the diluted pre-selective PCR product were used as selective amplification in a reaction tube containing 20 µl selective amplification mixtures (**Table 4.6**). Eight primer pairs were used for the selective amplification (**Table 4.3**). Selective amplification mixtures were performed the following selective PCR program. The selective amplification product was added with 10 µl of sequencing dye. The selective amplified PCR products were determined by using 1% agarose gel electrophoresis in 0.5x TBE. The selective PCR products were run on 4.5% denaturing polyacrylamide gel electrophoresis.

Table 4.6 Reaction mixture for selective amplification reaction.

Ligation components	**Volume (µl)**
Dilute pre-amplification product	3.00
10 µM ER3A_ _ (Eurofins MWG Operon, Germany)	1.00
10 µM MS3C_ _ (Eurofins MWG Operon, Germany)	1.00
10x PCR buffer (Invitrogen, USA)	2.00
50 mM $MgCl_2$ (Invitrogen, USA)	1.00
10 mM dNTPs (Fermentas, Canada)	0.40
5 U/µl Taq polymerase (Invitrogen, USA)	0.10
ddH_2O	11.00
Total	20.00

Selective amplification PCR program (C1000 Thermal Cycler, Biorad)

Step 1:	Predenaturation step	95°C	3 min.
Step 2:	Denaturation step	95°C	30 sec.
Step 3:	Annealing step	65°C	30 sec. (starting at 65 °C and reducing each step 0.6 °C until 56 °C)
Step 4:	Extension step	72°C	1 min
Step 5:	12 cycles; step 2 to step 4		
Step 6:	Denaturation step	95°C	30 sec
Step 7:	Annealing step	56°C	30 sec
Step 8:	Extension step	72°C	1 min
Step 9:	23 cycles; step 6 to 8		
Step 10:	Final extension step	72°C	10 min
Step 11:	Hold	4°C	∞
Step 12:	End		

4.2.3 Detection of AFLPs bands using denaturing polyacrylamide gel electrophoresis

Selective amplification products (AFLPs products) were separated by 4.5% denaturing polyacrylamide gel electrophoresis in 0.5X TBE in a Sequi-Gen GT Sequencing cell (Biorad, USA). The AFLPs bands on polyacrylamide gel were detected by silver nitrate staining. The silver nitrate staining was followed the protocol described by Bassam *et al.* (1991).

4.2.4 Data analysis

For genetic diversity analysis, a band was considered polymorphic if it was present in at least one genotype and absent in others. AFLP amplification products were scored for their presence (1) or absence (0) across 17 accessions for all the primer combinations employed to generate binary matrix. Genetic similarity (GS) was calculated by making a pairwise comparison among the accession using Jaccard's coefficient (Jaccard, 1908).The formula is given as follows: $Gs_{ij} = a/a + b + c$, where Gs_{ij} is the measure of GS between individuals i and j, a is the number of polymorphic fragments are shared by i and j, b is the number of fragments present in i and absent in j, and c is the number of fragments present in j and absent in i. A dendrogram was constructed from the matrix of similarity coefficients using the Unweighted Pair-Group Method of the Arithmetic average (UPGMA) technique (Sneath and Sokal, 1973) of the NTSYS-pc software package version 2.11T (Rohlf, 2000). The cophenetic correlation coefficient was generated by means of COPH routine in order to check the goodness of fit between the cluster in the dendrogram and the similarity coefficient matrix.

4.3 Results

4.3.1 AFLP analysis

Genomic DNA was extracted from leaves of each sample using a CTAB extraction method. The quality of extracted genomic DNA was electrophoretically determined using 0.8% agarose gel (w/v). High molecular weight DNA at 23.1 Kbs along with sheared DNA was obtained from that leaves. Some DNA samples contained RNA contamination as visualized by the smear at the bottom of gel.

A total of 28 primer combinations were screened, and the amplification products of eight primer combinations (**Table 4.7**) were further genotyped across all investigated taxa. In total, 461 amplified fragments ranging from 40 to 700 bps in size were generated from respective primer combinations. The average number of polymorphic bands per primer was 58, while the amounts for the eight primers range from 24 to 107 bands (**Table 4.7**). The AFLP profiles can be used to distinguish between the *Lagerstroemia* accessions by their unique band patterns (**Figure 4.1 – 4.8**).

Table 4.7 Primer combination, the number of AFLP bands, size range and the percentage of polymorphic bands resulted from AFLP analyses of this study.

Primer combination	Number of AFLP bands	Size range (bps)	Percentage of polymorphic bands
ER3AAC / MS3CAG	88	40 – 700	100
ER3AAC / MS3CAT	67	40 – 700	98
ER3AAC / MS3CTT	24	60 – 600	100
ER3AAG / MS3CTG	54	40 – 700	96.30
ER3ACC / MS3CAC	43	40 – 600	100
ER3ACC / MS3CAT	51	40 – 700	96.08
ER3ACC / MS3CTA	27	60 – 600	100
ER3ACC / MS3CTT	107	40 – 700	100
Total	461	40 – 700	98.9795

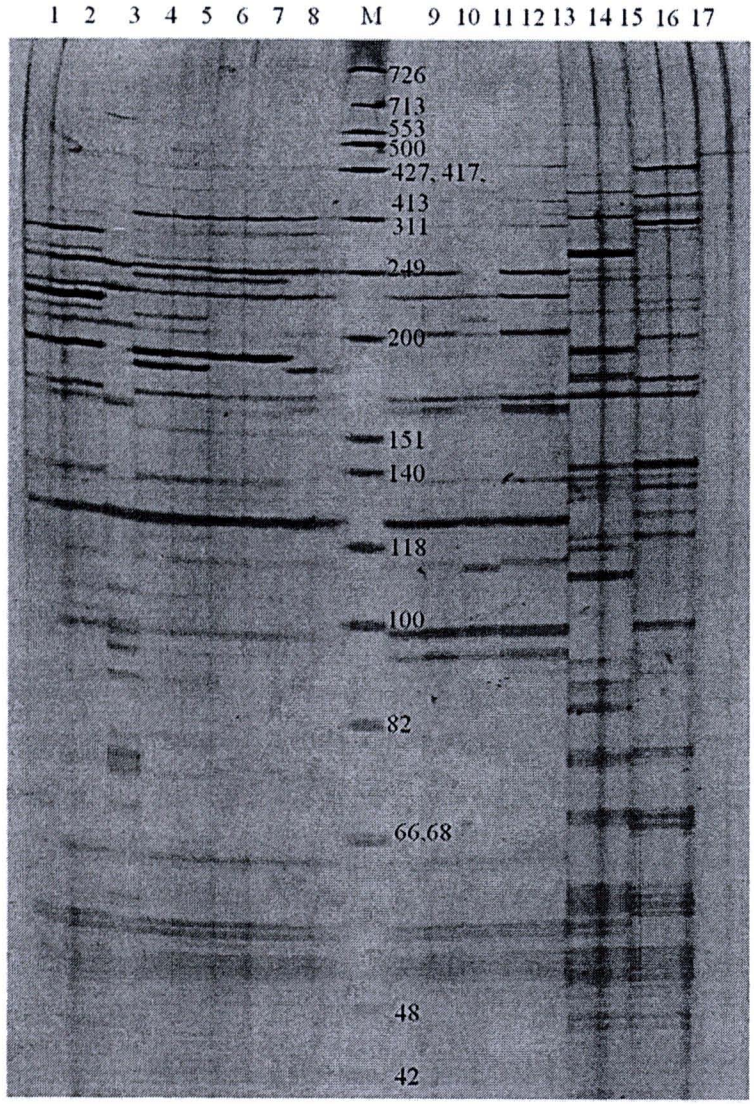

Figure 4.1 AFLP profile generated by primer combination of ER3AAC, MS3CAG.

Lane designations with accession number in parenthesis are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1

Lane 2: *L. speciosa* sample LS2

Lane 3: *L. speciosa* sample LS3

Lane 4: *L. speciosa* sample LS4

Lane 5: *L. speciosa* sample LS5

Lane 6: *L. speciosa* sample LS6

Lane 7: *L. speciosa* sample LS7

Lane 8: *L. speciosa* sample LS8

Lane 9: *L. macrocarpa* sample LM1

Lane 10: *L. macrocarpa* sample LM2

Lane 11: *L. macrocarpa* sample LM3

Lane 12: *L. macrocarpa* sample LM4

Lane 13: *L. loudonii* sample LL1

Lane 14: *L. loudonii* sample LL2

Lane 15: *L. floribunda* sample LF1

Lane 16: *L. floribunda* sample LF2

Lane 17: *Lawsonia inermis* sample LAW

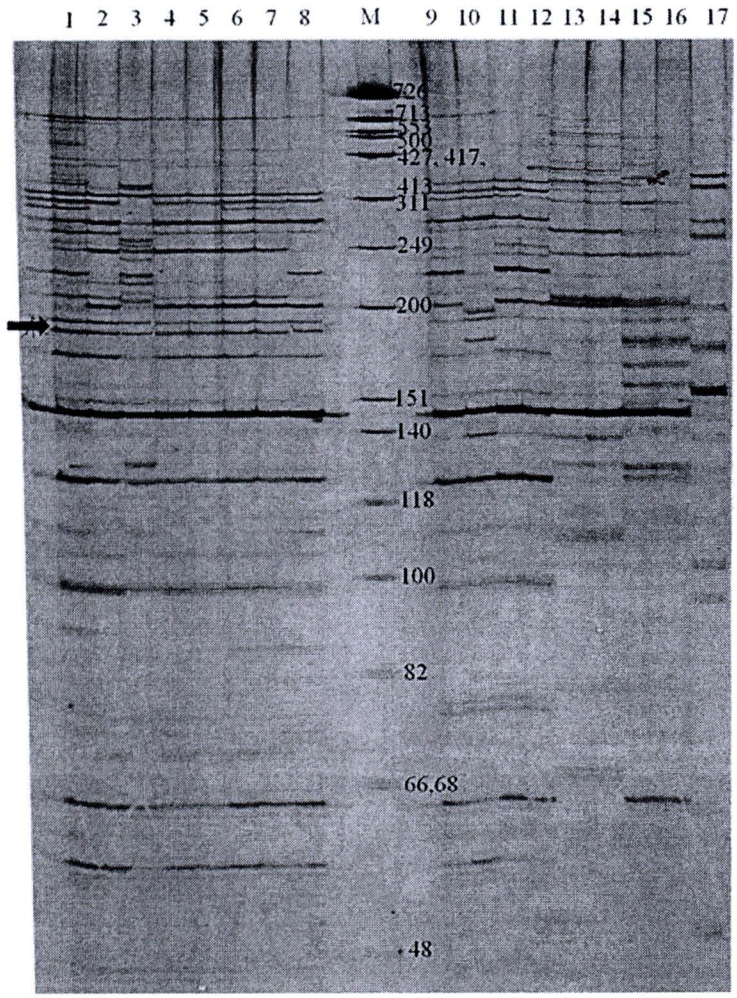

Figure 4.2 AFLP profile generated by primer combination of ER3AAC, MS3CAT. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1 Lane 2: *L. speciosa* sample LS2

Lane 3: *L. speciosa* sample LS3 Lane 4: *L. speciosa* sample LS4

Lane 5: *L. speciosa* sample LS5 Lane 6: *L. speciosa* sample LS6

Lane 7: *L. speciosa* sample LS7 Lane 8: *L. speciosa* sample LS8

Lane 9: *L. macrocarpa* sample LM1 Lane 10: *L. macrocarpa* sample LM2

Lane 11: *L. macrocarpa* sample LM3 Lane 12: *L. macrocarpa* sample LM4

Lane 13: *L. loudonii* sample LL1 Lane 14: *L. loudonii* sample LL2

Lane 15: *L. floribunda* sample LF1 Lane 16: *L. floribunda* sample LF2

Lane 17: *Lawsonia inermis* sample LAW

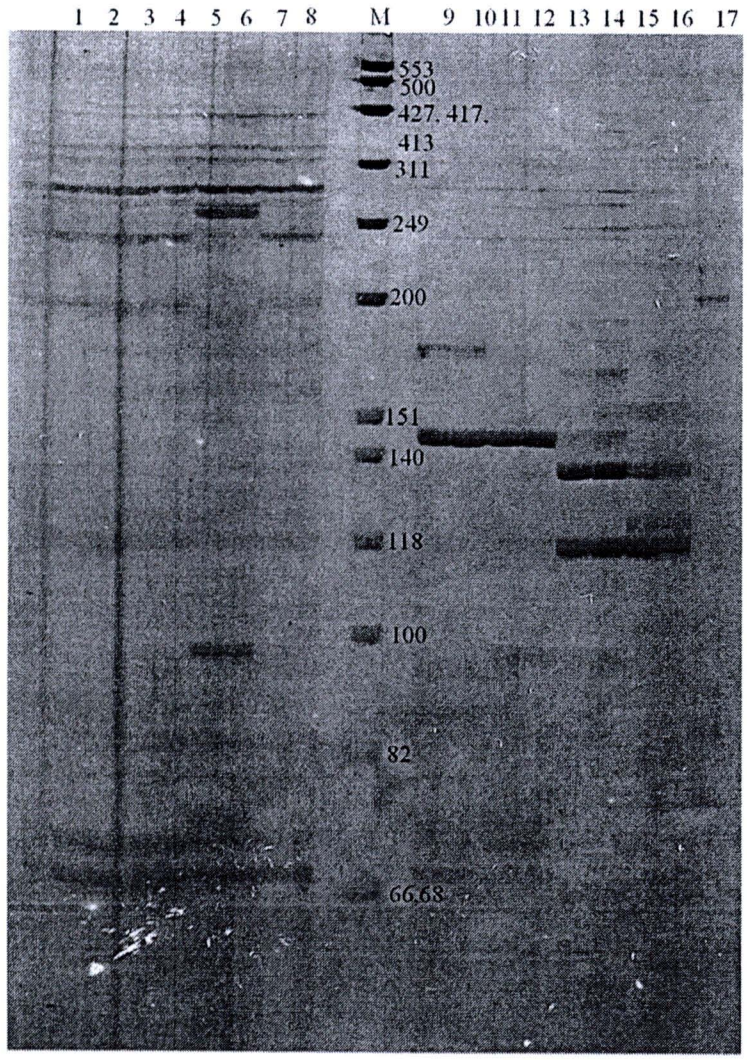

Figure 4.3 AFLP profile generated by primer combination of ER3AAC, MS3CTT. Lane designations with accession number in parenthesis are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1 Lane 2: *L. speciosa* sample LS2

Lane 3: *L. speciosa* sample LS3 Lane 4: *L. speciosa* sample LS4

Lane 5: *L. speciosa* sample LS5 Lane 6: *L. speciosa* sample LS6

Lane 7: *L. speciosa* sample LS7 Lane 8: *L. speciosa* sample LS8

Lane 9: *L. macrocarpa* sample LM1 Lane 10: *L. macrocarpa* sample LM2

Lane 11: *L. macrocarpa* sample LM3 Lane 12: *L. macrocarpa* sample LM4

Lane 13: *L. loudonii* sample LL1 Lane 14: *L. loudonii* sample LL2

Lane 15: *L. floribunda* sample LF1 Lane 16: *L. floribunda* sample LF2

Lane 17: *Lawsonia inermis* sample LAW

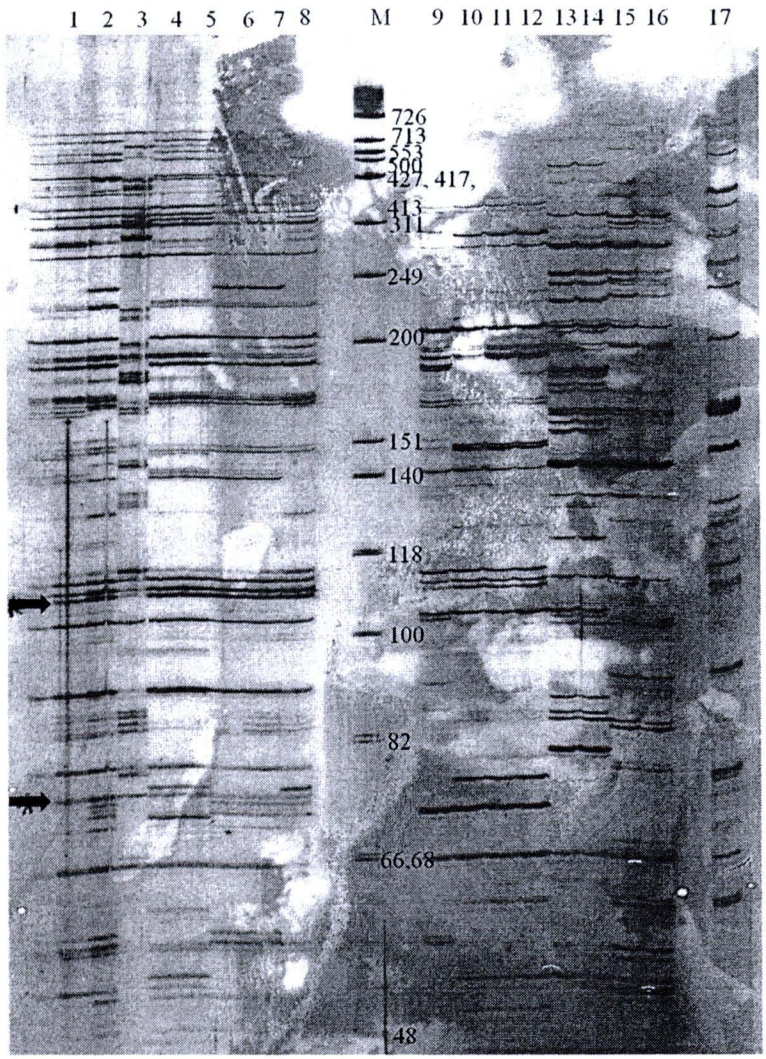

Figure 4.4 AFLP profile generated by primer combination of ER3AAG, MS3CTG. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

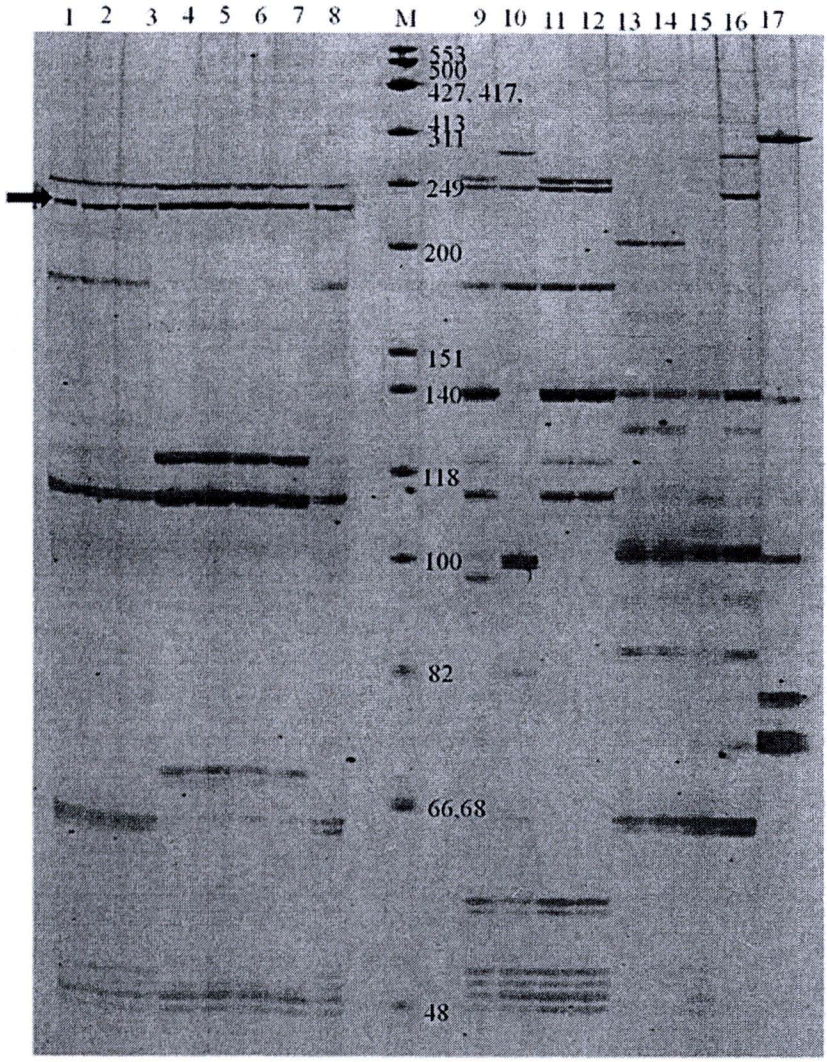

Figure 4.5 AFLP profile generated by primer combination of ER3ACC, MS3CAC. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

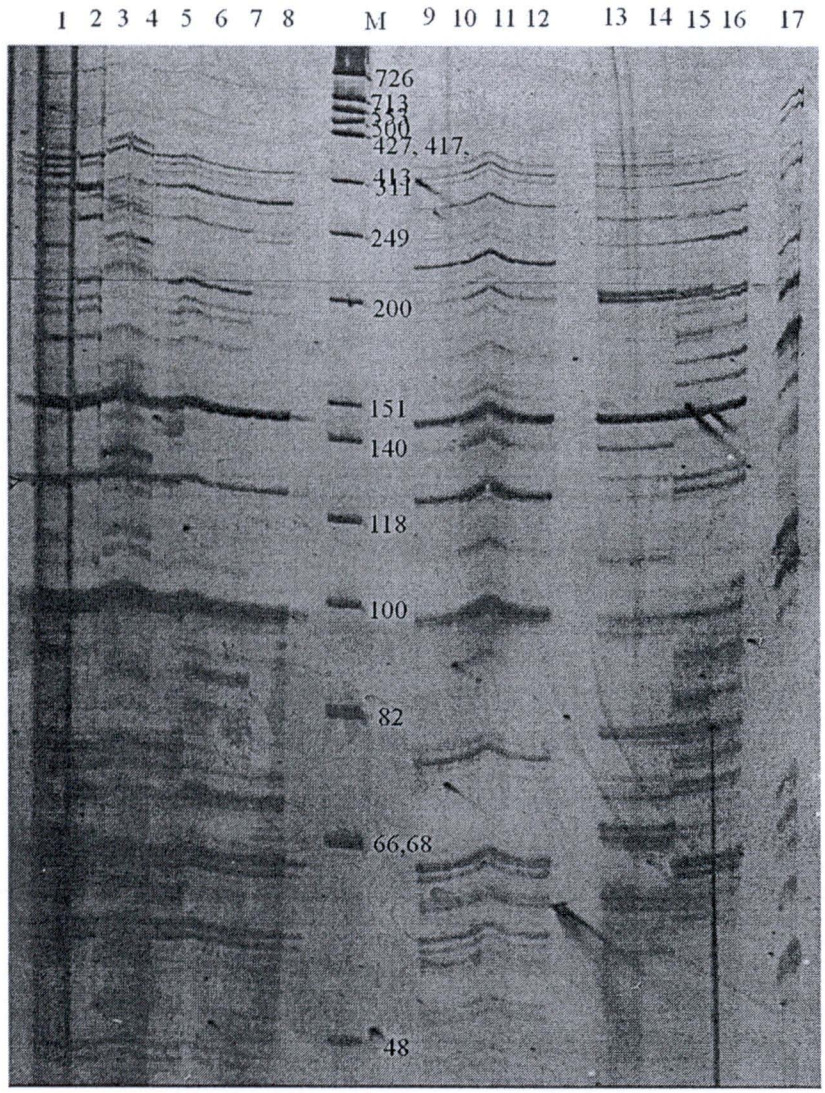

Figure 4.6 AFLP profile generated by primer combination of ER3ACC, MS3CAT. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

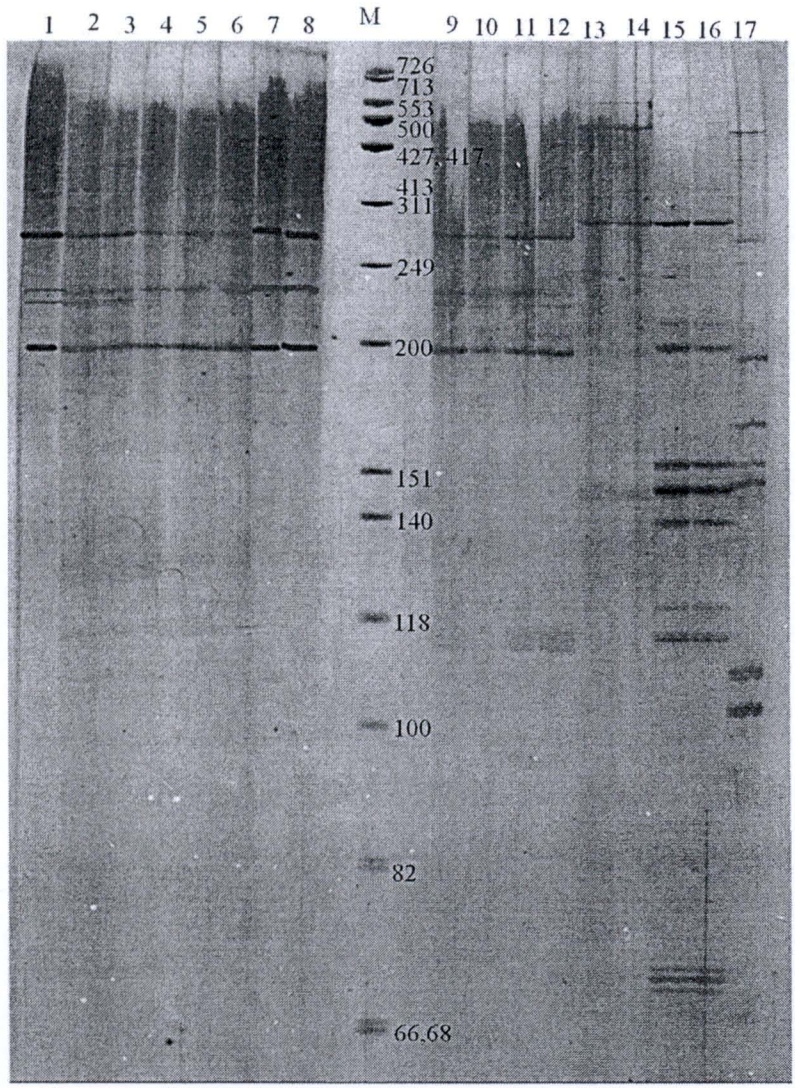

Figure 4.7 AFLP profile generated by primer combination of ER3ACC, MS3CTA. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

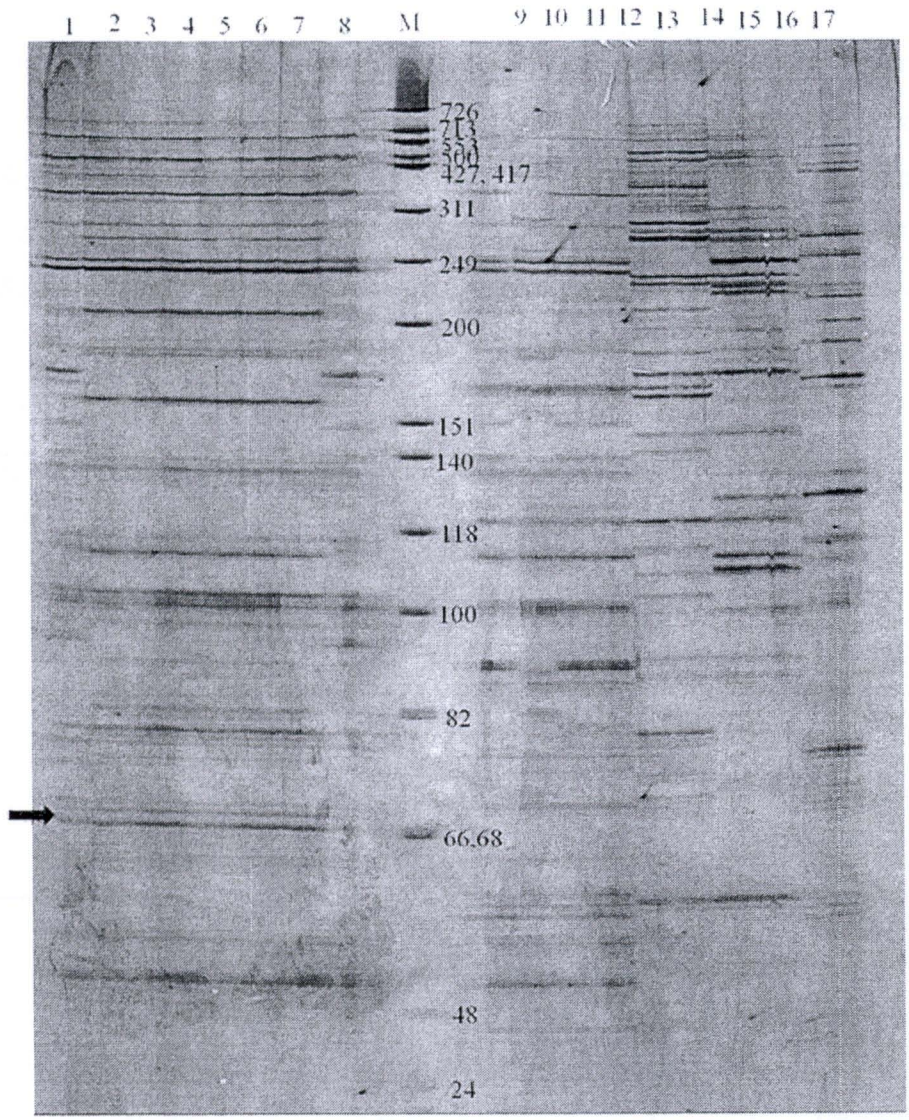

Figure 4.8 AFLP profile generated by primer combination of ER3ACC, MS3CTT. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

4.3.2 Genetic relationship

The dendrogram was generated by the Jaccard's similarity matrix and UPGMA method revealed genetic relationship among four *Lagerstroemia* species and outgroup, *Lawsonia* (**Figure 4.9**). A high cophenetic correlation coefficient of 0.99837 between the Jaccard's similarity data matrix and the cophenetic matrix was obtained, indicating a good fit between the dendrogram clusters and the similarity matrices. According to the dendrogram, four main groups were produced. Cluster I was composed of *L. speciosa* and *L. macrocarpa*. This cluster was divided into two subgroups. *L. loudonii* were in cluster II, whereas, *L. floribunda* were in cluster III. The outlier, *Lawsonia inermis*, was classified in cluster IV.

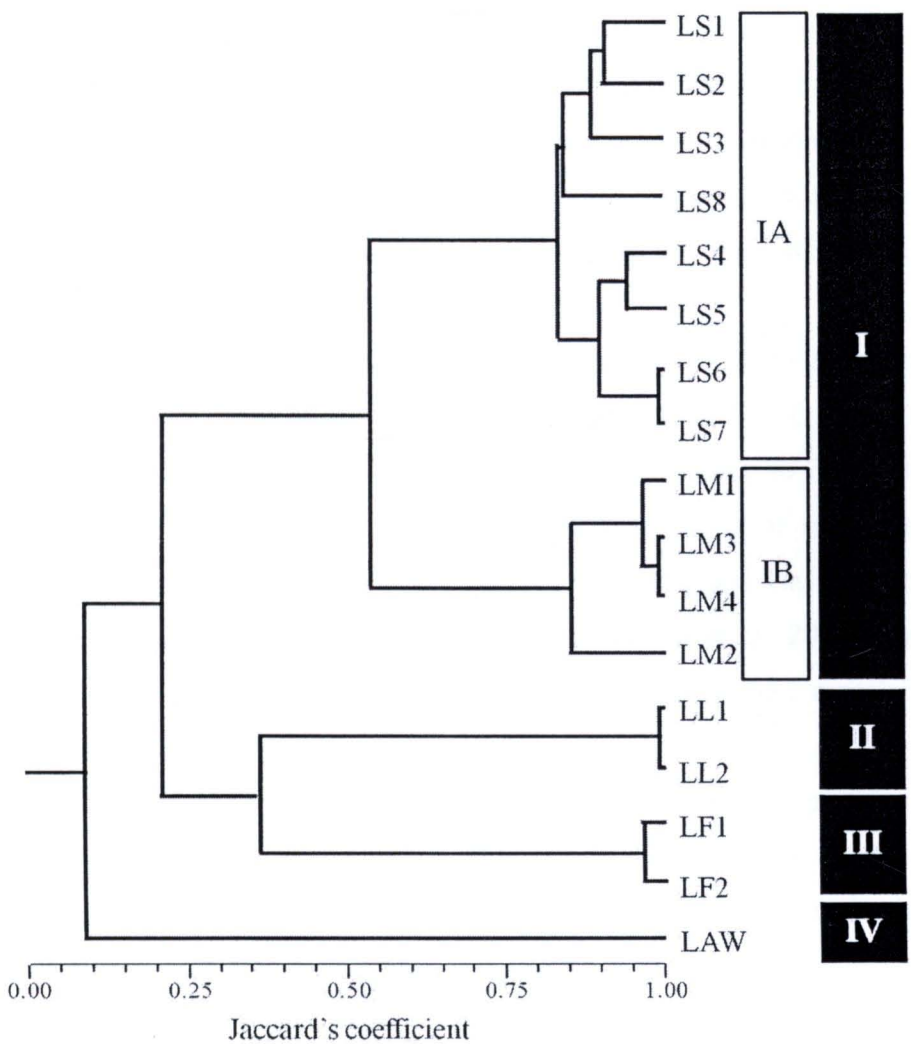

Figure 4.9 UPGMA dendrogram based on Jaccard's similarity coefficient among *Lagerstroemia* and *Lawsonia inermis* accessions.

4.4 Discussion

Assessment of genetic diversity provides an efficient and effective way to estimate genetic variation and delineate phenetic relationships among accessions. DNA-based markers, such as RAPD (Williams *et al.*, 1990), AFLP (Vos *et al.*, 1995) and SSR markers (Tautz *et al.*, 1984) have been routinely employed for analysis of variation in gene pool of herb and for medicinal plant identification. These molecular markers uncover variation at different genomic location (Powell *et al.*, 1996; Russel *et al.*, 1997). AFLP is based on selective PCR amplification of restriction fragments from a digest of total genomic DNA using PCR. The procedure of the AFLP analysis is more time-consuming than RAPD. However, a major advantage of AFLP markers is the capacity to reveal many polymorphic bands in one lane compared to RAPD markers (Barker *et al.*, 1999; Ha *et al.*, 2002). Because of the higher sensitivity of AFLP, the weak divergence can be efficiently detected by AFLP. Also, AFLP technique is a reliable, stable, and rapid assay for use in molecular marker screening (Jia *et al.*, 2001). Thus the AFLP technique has been increasingly used to assess genetic diversity and authentication in a variety of organisms (Cresswell *et al.*, 2001; Das *et al.*, 1999; Winfield *et al.*, 1998).

Three candidates species-specific of *L. speciosa*, a 230 bp fragment from ER3ACC/MS3CAC, a 110 bp fragment from ER3AAG/MS3CTG, and 190 bp fragment from ER3AAC/MS3CAT, should be developed further to SCAR marker. Conversion of AFLP marker to SCAR marker by sequencing of species-specific bands can provide effective methods for the identification and classification in a various species (He *et al.*, 2008; Kwon *et al.*, 2009; Radisek *et al.*, 2004; Sun *et al.*, 2005). Compared with AFLP markers, SCAR markers are stable, repeatable and convenient in practical application.

To the best of our knowledge, this is the first report on the application of the AFLP technique in the evaluation of the genetic relationship in the genus *Lagerstroemia* species. The dendrogram constructed on the basis of genetic similarity showed that all the *Lagerstroemia* accessions clustered in a way that is generally in agreement with the classification based on morphological characteristics.

The genetic similarity coefficient between *L. speciosa* and *L. macrocarpa* was 0.54, and they were clustered in a clade. The result suggested that these two species are closely related with other and they are very similar in morphological

characteristics. They all had monomorphic stamens with monomorphic pollen (Kim *et al.*, 1994). Compared with *L. speciosa*, *L. macrocarpa* had bigger fruit length (2.5–4 cm vs 1.5–2.5 cm) and flower size (5–7.5 cm vs. 6–10 cm) (Gardner *et al.*, 2007).

In this study, the dendrogram placed *L. loudonii* in clase II whereas *L. floribunda* was formed alone in clade III. The morphological characteristics displayed the softly grayish hairs below the leaves especially when it was young (Gardner *et al.*, 2007). *L floribunda* bark showed pale cream in color with smooth and flaking in thin plates (Gardner *et al.*, 2007).

The present contribution shows that AFLP is a promising approach to help in plant identification with three candidate species-specific primer combinations, ER3ACC/MS3CAC, ER3AAG/MS3CTG, and ER3AAC/MS3CAT, for *L. speciosa*. For further research, SCAR marker should be developed for identification of *L. speciosa*.