Amplified Fragment Length Polymorphism Analysis for Studying Genetic Relationships among *Mangifera* Species in Thailand

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ABSTRACT. The phylogenetic relationships among 14 *Mangifera* L. species including three economically important species, i.e., common mango (*M. indica* L.), horse mango (*M. foetida* Lour.) and kwini (*M. odorata* Griff.), were analyzed by comparing 217 amplified fragment length polymorphism (AFLP) markers. The unweighted pair grouping method using arithmetic averages (UPGMA) and neighbor-joining (NJ) method were used and two outgroup taxa, cashew nut (*Anacardium occidentale* L.) and gandaria (*Bouea macrophylla* Griff.), were added to both analyses. The common mango was closely related to banana mango (*M. sylvatica* Roxb.), *M. laurina* Bl., and *M. oblongifolia* Hook.f. Intraspecific variation among seven cultivars of common mango was much smaller than interspecific variation and these cultivars were classified into one *M. indica* group using both methods. *Mangifera macrocarpa* Bl., *M. foetida*, and *M. odorata* were also related to *M. indica* in both UPGMA and NJ trees, although these three species are classified into a different subgenus (subgenus *Limus*) from the subgenus *Mangifera* to which *M. indica* belongs. Also, in both UPGMA and NJ trees, *M. gedde* Miq. and *M. griffithii* Hk.f. were placed in distant positions among the *Mangifera* species tested, indicating these two species are related distantly to *M. indica*. The AFLP technique was confirmed to be useful for phylogenetic analysis.

The genus *Mangifera* is indigenous to southeast Asia, and mango (*Mangifera indica*) is the most important species in this genus. India was the first country to cultivate mango as a fruit crop, but currently, it is grown commonly throughout tropical areas of the world. Several botanists (Hou, 1978; Mondal et al., 1982; Mukherjee, 1953) have proposed classifications of the genus *Mangifera*. The most recent and acceptable taxonomic treatment is that by Kostermans and Bompard (1993) who described 69 species in the genus *Mangifera* based on morphological characteristics and classified 58 species into two subgenera with several sections. The position of the other 11 species was uncertain (Kostermans and Bompard, 1993). Such classical taxonomy is sometimes ambiguous and more reliable marker systems based on molecular biology are needed for modern taxonomy. Previously, we performed restriction site analysis of an amplified region of cpDNA of 13 *Mangifera* species (Eiadthong et al., 1999b), but could not obtain reasonable phylogenetic relationships among them due to a low level of polymorphism. Our current exploration of *Mangifera* species in Thailand for germplasm collection could benefit from application of molecular markers to determine the phylogenetic relationships among them.

Detection of polymorphism among germplasm collections for selected species will provide insight into the genome evolution, origin of cultivated species, and current level of diversity in modern agricultural crops (Becker et al., 1995; Hill et al., 1996).

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Amplified fragment length polymorphism (AFLP) analysis is one of the most recent innovations in genetic marker technologies and provides greater sensitivity for detecting a high level of polymorphism; for example, in coconut (*Cocos nucifera* L.) (Perera et al., 1998), cassava (*Manihot esculenta* Crantz) (Roa et al., 1997), rice (*Oryza sativa* L.) (Zhu et al., 1998), and maize (*Zea mays* L.) (Marsan et al., 1998). This technique is based on selective, semiquantitative polymerase chain reaction (PCR) amplification of restriction fragments digested from total genomic DNA. In this technique, fragments generated by digestion of DNA with a combination of two restriction endonucleases are ligated to suitable adapters and, thereafter, ligated DNA fragments are amplified selectively with different primer combinations (Vos et al., 1995). Therefore, the objectives of the present study were to 1) evaluate the effectiveness of AFLP analysis for detecting the genetic diversity in mango and its wild relatives and 2) examine phylogenetic relationships among *Mangifera* species from the result of AFLP analysis.

Materials and Methods

PLANT MATERIALS. All 14 species used in this study (Table 1) were collected in Thailand during 1995–97 from several survey trips (Eiadthong et al., 1999a). Seven cultivars of common mango and two accessions each of *M. flava*, *M. caloneura*, *M. odorata*, *M. pentandra*, and *M. sylvatica* were included in the analysis to examine intraspecific diversity. The eight remaining species were analyzed for only one accession because no other accessions were available. Two other genera of Anacardiaceae, cashew nut (*Anacardium occidentale* L.) and gandaria (*Bouea macrophylla* Griff.), also were included as outgroup taxa in this study.

DNA EXTRACTION AND AFLP ANALYSIS. Total DNAs were ex-

Table 1. List of 14 *Mangifera* species and two outgroup taxa with their collected locations for AFLP analysis.

Scientific name	Common name	Collected locations (voucher specimen no.)
<i>M. caloneura</i> Kurz	Mamuang Kalon paa ^z	Sakaerat Ecological Research Station, Nakon Ratchasima (MR-1); Sikhiu, Nakon Ratchasima (MR-2)
<i>M. cochinchinensis</i> Engler	Mamuang Khailaen ^z	Soidao, Chanthaburi (MCC-1)
<i>M. collina</i> Kosterm.	Mamuang Doi ^z	Doi Suthep National Park, Chiang Mai (MN-1)
<i>M. flava</i> Evrard	Paeb ^y	Khao Chong arboretum, Trang (MV-1; MV-2)
<i>M. foetida</i> Lour.	Horse mango	Baan Toun Nga Chang, Songkhla (MF-1)
<i>M. gedebe</i> Miq.	Mamuang paan ^z	Chalermprakiet Wildlife Sanctuary, Narathiwat (ME-1)
<i>M. griffithii</i> Hk.f.	Mamuang Rawa ^z	Sugai Padee, Narathiwat (MG-1)
<i>M. indica</i> L. ^y	Common mango	Pak Chong, Nakon Ratchasima ('Adam', 'Irwin', 'Raed', 'Pim Sen Man', 'Nam Dok Mai' and 'Sampee'); Nonthaburi ('Salaya')
<i>M. laurina</i> Bl.	Mamuang Pruan ^z	Sikao, Trang (ML-1)
<i>M. macrocarpa</i> Bl.	Muang Kheekwang ^z	Khao Chong arboretum, Trang (MM-1)
<i>M. oblongifolia</i> Hk.f.	Mamuang Baap ^z	Soidao, Chanthaburi (MB-1)
<i>M. odorata</i> Griff.	Kwini	Pak Chong, Nakon Ratchasima (MO-1); Chana, Songkhla (MO-2)
<i>M. pentandra</i> Hk.f.	Muang Kan ^z	Takbai, Narathiwat (MP-1; MP-2)
<i>M. sylvatica</i> Roxb.	Banana mango	Thong Phaphum, Kanchanaburi (MS-1); Sikao, Trang (MS-2)
<i>Anacardium occidentale</i> L.	Cashew nut	Pak Chong, Nakon Ratchasima (AN)
<i>Bouea macrophylla</i> Griff.	Gandaria	Sugai Padee, Narathiwat (B)

^zLocal name in Thai.^yAll cultivars of *M. indica* were not numbered.

tracted from the leaves of each plant by the CTAB method of Doyle and Doyle (1987) with a slight modification (Eiadthong et al., 1999b). The precipitated DNAs were then dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA (TE buffer) and were purified by extraction with a mixture of 25 phenol : 24 chloroform : 1 isoamyl alcohol. After DNA concentrations were adjusted to ≈ 20 ng- μ L⁻¹ by comparison with standard λ -DNA on a minigel (Sambrook et al., 1989), the AFLP reactions were performed following the manufacturer's instructions in the AFLP analysis system I (Life Technologies, Gaithersburg, Md.) manual. Briefly, ≈ 250 ng genomic DNA was digested with 2.5 units *Eco*RI and 2.5 units *Mse*I at 37 °C for 3 h and incubated at 70 °C for 15 min. The DNA fragments were ligated to *Eco*RI and *Mse*I adapters at 20 °C for 3 h. After preselective amplification, selective amplification was performed with 64 combinations of *Eco*RI and *Mse*I primers provided by Life Technologies.

AFLP reactions were denatured by boiling with formamide buffer (98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol) and loaded on a 6% denaturing polyacrylamide gel (15 $\times$ 20 cm). All samples were electrophoresed for 6 h at 200 V and then transferred to a Biotodyne B membrane (Pall, Inc., East Hills, N.Y.) by capillary blotting overnight. The membrane was hybridized with the AFLP nonradioactive probe (Life Technologies) by following the manufacturer's instructions, and amplified fragments were detected after incubation with CDP-Star (Boehringer Mannheim, Germany) at 37 °C for 15 min and exposure to X-ray film for 3 h. AFLP samples of *M. indica* 'Irwin' and 'Adam', were always included in each run of the gel as references for determining the corresponding bands for data analysis.

DATA ANALYSIS. A binary matrix reflecting the presence (1) or absence (0) of each AFLP band was generated for each genotype. After similarity indices (Nei and Li, 1979) were calculated based on the number of shared bands, the unweighted pair grouping method using arithmetic averages (UPGMA) was used to construct a dendrogram with NTSYS-pc ver. 2.01 (Rohlf, 1997). A second dendrogram was produced using the neighbor-joining (NJ) method (Saitou and Nei, 1987) executed through PHYLIP (Felsenstein, 1993).

Results and Discussion

SELECTIVE PRIMER COMBINATIONS FOR AFLP ANALYSIS. Based on the number of scorable bands and the levels of polymorphism detected by screening two cultivars of *M. indica* ('Irwin' and 'Adam'), one accession of *M. caloneura*, and one accession of *M. foetida* with 64 primer combinations, five primer combinations were selected for further analysis (Table 2). The reproducibility of amplified bands was confirmed with repeated reactions using the same template DNAs and primer combinations selected from the preliminary screen. By the AFLP analysis of these five primer combinations, a total of 217 scorable bands were detected of which 77% were polymorphic among *Mangifera* species (Table 2). The highest number of polymorphic bands among *Mangifera* species was obtained from the E-AAC/M-CAG primer combination. AFLP banding patterns of four species produced by each primer combination are shown in Fig. 1. The dendrogram of 14 *Mangifera* species was constructed using these 217 scorable bands by cluster analysis using UPGMA and the NJ method for phylogenetic analysis.

PHYLOGENETIC RELATIONSHIPS. Seven cultivars of *M. indica* were clustered into one group by both UPGMA and NJ methods (Figs. 2 and 3). Each of the two accessions of *M. odorata*, *M. caloneura*, *M. pentandra*, *M. flava*, and *M. sylvatica* also formed

Table 2. Number of scorable and polymorphic bands produced by five primer combinations from 14 *Mangifera* species.

Primer combination	No. of scorable bands	No. of polymorphic bands among <i>Mangifera</i> sp.	Polymorphic bands (%)
E-AAC/M-CAC	42	32	76
E-AAC/M-CAG	54	50	93
E-AAC/M-CTC	58	43	74
E-AAC/M-CTG	36	22	61
E-ACC/M-CTC	27	20	74
Total	217	167	77

Fig. 1. AFLP profiles of four species including two accessions of one species produced by selected five primer combinations. Primer combination is shown at upper portion of lane number. Lane 1, *M. indica* 'Nam Dok Mai'; lane 2, *M. sylvatica* (MS-1); lane 3, *M. flava* (MV-1); lane 4, *M. flava* (MV-2); and lane 5, *M. laurina*.

intraspecific groups. The intraspecific polymorphisms were less abundant than interspecific polymorphisms in AFLP analysis. These results imply that AFLP analysis is an efficient and reliable technique to generate data for either interspecific or intraspecific phylogenetic study in the genus *Mangifera*. An error source in AFLP analysis for phylogenetic study could be the nonhomology of comigrating fragments in the gel, the same issue as with RAPD markers, which was discussed by Karp et al. (1996) and Spooner et al. (1996). When analyzing a relatively large genome, too many bands produced by some AFLP primer pairs might increase the problem of comigrating fragments. However, the possibility having a similar AFLP fragment of apparently identical molecular weight in different individuals seems to be minimized with AFLPs compared to RAPD markers due to the highly selective amplification of a small subset of the whole genome

and the more sensitive separation range (Kardolus et al., 1998; Mace et al., 1999). In fact, Rouppe van der Voort et al. (1997) reported that 19 of 20 comigrating AFLP markers were identical. Furthermore, the occurrence of overlapping bands from different loci may not cause any serious problem in estimations of variation (Hansen et al., 1999). The occurrence of overlapping bands from different loci results in a downward bias, the accessions appearing to be more closely related than they actually are. However, since it is relative rather than absolute in genetic distances, the bias is not expected to cause any serious distortion in establishing genetic relationships (Hansen et al., 1999).

Dendrograms produced using the UPGMA and NJ methods showed similar relationships among the 14 *Mangifera* species. In both UPGMA and NJ trees, seven species formed a primary cluster that included common mango. The species most closely related to *M. indica* were *M. laurina* and *M. sylvatica* followed by *M. oblongifolia*, *M. odorata*, *M. macrocarpa*, and *M. foetida* (Figs. 2 and 3). The only major discrepancy between the two analyses was in placement of *M. cochinchinensis*. According to the NJ tree, *M. cochinchinensis* was positioned in a cluster along with *M. gedebe*, *M. griffithii*, and the two out group taxa. Although UPGMA cluster analysis also placed *M. gedebe* and *M. griffithii* at a distant position among *Mangifera* species along with the two outgroup taxa (Fig. 2), *M. cochinchinensis* fell in a cluster with *M. collina*, *M. pentandra*, *M. flava*, and *M. caloneura* (Fig. 2).

Kostermans and Bompard (1993) divided the genus *Mangifera* into two subgenera, *Mangifera* and *Limus* based on flower morphology. The subgenus *Mangifera* has four sections, section *Marchandora*, section *Euantherae*, section *Rawa*, and section *Mangifera*, while the subgenus *Limus* has two sections, section *Deciduae* and section *Perennes* (Kostermans and Bompard, 1993). Among the species analyzed in the present study, *M. gedebe* has been assigned to the section *Marchandora*, *M. griffithii* belongs to the section *Rawa*, and *M. pentandra*, *M. caloneura*, and *M. cochinchinensis* belong to the section *Euantherae*. *Mangifera indica*, *M. oblongifolia*, *M. collina*, *M. laurina*, *M.*

Fig. 2. UPGMA cluster analysis for 14 *Mangifera* species including 7 mango cultivars and accessions of some species based on 217 AFLP markers.

its use as a synonym of *M. indica* (Kochummen, 1996). *Mangifera laurina* is thought to be immune to anthracnose caused by *Colletotrichum gloeosporioides* Penz. (Bompard and Schnell, 1997) and a good material for breeding disease resistance into mango, even though the fruit is very sour. Also, this species is used occasionally as a rootstock for commercial mango cultivars on periodically inundated riverbeds (Bompard, 1993). The close genetic relationship of this species to *M. indica* indicates that these traits may be transferred to common mango (*M. indica*) for commercial production. On the other hand, evidence that *M. sylvatica* may have been involved in development of cultivated *M. indica* has been shown by RFLP analysis of an amplified region of cpDNA (Eiadthong et al., 1999b). The fruit of *M. sylvatica* is sweeter and juicier, although smaller, than that of common mango. This species may also have a potential value for mango breeding.

Mangifera gedebe and *M. griffithii* are grown in swamps and are thought to be promising sources of rootstock for development of mango cultivation on poorly drained soils and inundated lands (Ram, 1997). However, the distant position of these species from *M. indica* shown in the present analyses may constitute a problem for graft compatibility, although no references are available for it. The close relationship of *M. odorata* and *M. foetida* to common mango revealed in the present analyses suggests a possibility for use of these species as breeding materials of common mango. In both species, the ripe fruit has a distinct and strong odor, and exhibits anthracnose resistance. These two species produce

larger fruit than common mango. If the assumption by Hou (1978) that *M. odorata* may be a hybrid between common mango and *M. foetida* is true, these species may be cross-compatible with common mango.

Another result from our analyses questions the position of the two outgroup taxa. The morphological characteristics of *gandaria* are closer to those of the genus *Mangifera* than cashew nut. This species was placed formerly in the genus *Mangifera* as *M. gandaria* Roxb. (Kostermans and Bompard, 1993). However, according to our analyses, the phylogenetic position of this species was more distant from *Mangifera* species than cashew nut (Figs. 2 and 3). Again, further analysis using molecular tools apart from morphology, is required in this case.

In summary, reliable biosystematic data for evaluating phylogenetic relationships among *Mangifera* may be produced rapidly using the AFLP technique. In addition, AFLP markers may be useful for evaluating the diversity of germplasm collections of *Mangifera* species.

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Fig. 3. PHYLIP neighbor-joining tree for 14 *Mangifera* species including 7 mango cultivars and accessions of some species based on 217 AFLP markers. Arm-length corresponds with dissimilarity index from node.

flava, and *M. sylvatica* belong to the section *Mangifera*. All these 11 species belong to the subgenus *Mangifera*. The remaining three species, *M. foetida*, *M. macrocarpa*, and *M. odorata*, belong to the section *Perennes* of the subgenus *Limus*.

According to our analyses, *M. foetida*, *M. macrocarpa*, and *M. odorata* which were placed into the subgenus *Limus* by Kostermans and Bompard (1993), instead belong to the section *Mangifera* along with *M. indica*, *M. laurina*, *M. sylvatica*, and *M. oblongifolia*. Also, the taxonomic position of *M. collina* and *M. flava* which Kostermans and Bompard (1993) placed in the section *Mangifera* should be reconsidered since our molecular analysis suggested they were more closely related to species in the section *Euantherae* including *M. pentandra* and *M. caloneura*. The classification of the nine other species, however, was in agreement with that proposed by Kostermans and Bompard (1993), although the position of *M. cochinchinensis* in the NJ tree was slightly questionable in our analyses.

A close relationship of *M. laurina* and *M. sylvatica* to *M. indica* was clearly revealed in the present analyses. The close relationship of *M. laurina* to *M. indica* may also be inferred from

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