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Genetic diversity of *Elaeagnuslatifolia* L. as revealed by Random Amplified Polymorphic DNA (RAPD) markers

Pratoomporn Yingthongchai[1], Daruni Naphrom[2], and Prasartporn Smitamana[1*]

[1]Plant Biotechnology Program, Graduate School, Chiang Mai University, Chiang Mai, 50200, Thailand, [2]Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand

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Elaeagnuslatifolia L., an endemic fruit plant, is found in the upper north of Thailand. Sixty accessions were collected from seven locations: Chiang Mai, Chiang Rai, Lamphun, Lampang, Nan, Phayao and Mae Hong Son. Randomly amplified polymorphic DNA (RAPD) markers were used to identify genetic diversity of *E. latifolia*. Seven primers were screened from 50primers and provided a total of 178 DNA fragments of which 168 (94.38 %) were polymorphic. The genetic similarity matrix was calculated using the Jaccard similarity coefficient, and SAHN procedure was used for Dendrogram clustering. Jaccard's similarity coefficient(GSj) ranged from 0.38-0.71 and the cophenetic correlation of the similarity matrix (*r*) was 0.73. Fifty eight out of 60 accessions could be distinguished where as only two accessions; N55 and N72 appeared to be close to each other, with the genetic similarity coefficient of 0.71. The results indicated that the RAPD method is sufficiently informative and powerful to assess the genetic diversity in *E. latifolia*.

Keywords: *Elaeagnuslatifolia* L., DNA fingerprinting, Genetic diversity, RAPD markers

Introduction

The plants in the genus *Elaeagnus* (Family Elaeagnaceae) are distributed widely from the northern regions of Asia to the Himalayas, and different parts of Europe and North America. In India they grow up to an altitude of 2300 m in the Himalayas (Ahmadiani*et al.*, 2000; Bhattacharyya and Johri, 1998). Different species of *Elaeagnus* are cultivated as deciduous or evergreen ornamental shrubs. This genus is known to contain mono-and disaccharides in the leaves, fructose and L-ascorbic acid in the fruits, fatty acids and phytosterols in the seed, leaves and stems and carotenoids in the fruits (Sakamura and Suga, 1987).

*Corresponding author:PrasartpornSmitamana; e-mail: psporn@gmail.com

One species of this genus, *Elaeagnuslatifolia* L., is an endemic fruit plant mostly found in the upper north of Thailand. The plant has long been known by the local people and available in local markets where fruit were sold for about 10-15 Baht/kg. It is a climbing shrub rarely a tree; a scandent shrub with rusty shiny scales that are often thorny or spiny. Fruit is considered to be a very rich source of vitamins and minerals and other bioactive compounds. It is also a good source of essential fatty acids, which is unusual in other fruit trees. The tree is a great potential crop because of its good adaptability and toleration to different habitats, early bearing, high yield and various uses of ripe fruit. Fruit are eaten raw and used for making chutney, fruit pulp was used for making jam, jelly and refreshing drinks. The fruit of *E. latifolia* is being evaluated as a food that is capable of reducing the incidence of cancer and also as a means of halting or reversing the growth of cancers (Patel *et al*., 2008; Sundriyal and Sundriyal, 2003; Sundriyal and Sundriyal, 2005). *E.latifolia* is an actinorhizal plant (forms a symbiosis with the nitrogen-fixing bacterium *Frankia*)that has been studied in Kumuan Himalaya for its ability to restore degraded lands, and its leaves provide good source of fodder (Bargali, 2011).

In recent years, populations of *E. latifolia* have rapidly declined and the plant has disappeared from many locations of Thailand. The risk of the occurrence can be caused by the plant as endemic species and the fruits do not well known in the markets, therefore it is left out and only seasonal fruit picking without further proper propagation has been done. Moreover, the areas are replanted with the other more economically important crops. Therefore, characterization of the genetic diversity of *E. latifolia* is important for sustainable genetic conservation and increased use of plant genetic resources

Materials and methods

Plant materials

Sixty accessions of *E. latifolia*, were collected from seven provinces in the upper north of Thailand, i.e. Chiang Mai (CM), Chiang Rai (CR), Mae Hong Son (MH), Nan (N), Phayao (PY), Lamphun (L) and Lampang (LP) for RAPD analysis (Fig. 1 and Table 1). Two to three young leaves were removed from each tree and stored in freezers at -20ºC. until use.

DNA extraction

Total genomic DNA was extracted from approximately 100 mgyoung leaves using DNeasy Plant Mini kit (Qiagen) following the protocol of the manufacturer. The quantity and quality of isolated total genomic DNA was

determined using 0.8% agarose gel electrophoresis in 0.5X TBE buffer for mobility relative to know concentration of EZ load Precision Molecular Mass Standard and diluted to uniform concentration (10 ng/µl) for RAPD analysis.

DNA amplification

Sevenprimers were screened from 50primers) Operon Technology, USA). DNA amplification was carried out in 25 µl reactions consisting of pureTaq Ready-To-Go PCR Beads (GE Healthcare UK Limited, UK), 0.2 µM of each primer, 10 ng of template DNA and double-distilled water. The reaction was performed in Gradient Palm-Cycle™ (The Corbett Research Life Science, Australia). Amplification condition consisted of pre denaturation at 94 °Cfor 5 min, followed by 2 cycles of 94 °C for 60 sec, 38 °C for 7 sec, 72°C for 70 sec, and 55 cycles of 92 °C for 2 sec, 40 °C for 7 sec, 72 °C for 70 sec, followed by a final extension of 72 °C for 5 min. Amplification products were analyzed by electrophoresis at a constant voltage of 120 V for 2 h, in 1.5 % agarose gels with0.5X TBE buffer. A 100 bp ladder plus (Fermentas, USA) was used to estimate the molecular size of the fragments. After electrophoresis the gel was stained withethidium bromide (0.5µg/ml) and the patterns were photographed using a gel documentation system.

Data analysis

The observed bands in the gel were evaluated based on the presence (1) or absence (0) of polymorphic fragments for each primer. Only data from intensely stained, unambiguous, clearly visible bands were used for statistical analysis. Cluster analysis was performed with NTSYS-pc. Version 2.2, a numerical taxonomy and multivariate analysis software package(Rohlf, 2005) using unweighted pair-group method with an arithmetic average (UPGMA). Genetic similarities between samples were measured by the Jaccard's similarity coefficient (GSj) with SIMQUAL module. The distance coefficients were used to construct a dendrogram using UPGMA employing the Sequential Agglomerative Hierarchical and Nested (SAHN) algorithm. The goodness of fit of the clustering compared with the basic data matrix was tested by computing the cophenetic correlation coefficient using the normalized Mantel statistics Z test via the COPH and MXCOMP procedures. Polymorphic information content (PIC) was calculated for RAPD markers; PIC = $1-\sum P_{ij}^2$, where relative frequency of *j*th band for *i*th primer is summed across all the bands for the primer over all accessions (Li *et al*., 2009).

Fig. 1. *Elaeagnuslatifolia*L.

Table 1. Sixty accessionsof *Elaeagnus latifolia*L. used in RAPD analysis

Location	Number of accessions	Accessions code
Lamphun (L)	9	L2, L31, L42, L56, L57, L59, L65, L71, L72 (No. 1-No. 9)
Lampang (LP)	7	LP20, LP53, LP59, LP61, LP67, LP68, LP70 (No. 10-No. 16)
Mae Hong Son (MH)	9	MH5, MH31, MH49, MH54, MH60, MH63, MH70, MH72, MH74 (No. 17-No. 25)
Chiang Mai (CM)	11	H15, H19, H26, H31, H38, H46, MT12, MT16, D2, CM80, CM114 (No. 26-No. 36)
Nan (N)	7	N55, N72, N76, N99, N100, N101, N110 (No.37- No.43)
Phayao (PY)	7	PY16, PY52, PY56, PY62, PY63, PY68, PY85 (No. 44- No. 50)
Chiang Rai (CR)	10	CR29, CR66, CR70, CR90, CR98, CR103, CR104, CR108, CR113, CR119 (No. 51- No. 60)

Results

Out of 50 random decamer primers screened for polymorphism, 7 primers were selected for characterization of 60 accessions of *E. latifolia* (OPL-12, OPO-19, OPR-04, OPT-12, OPY-04, OPU-13,OPAJ-18andOPC-19). Amplification of genomic DNA of 60 accessions using 7 primers for RAPD analysis gave the results shown in Table 2. The number of fragments produced by each primer ranged from 18 (OPT-12) to 31 (OPO-19 and OPR-04). A total of 178 DNA fragments were identified, 168 of them were polymorphic with an average of 24.00 polymorphic fragments per primer. The percentage of

polymorphic bands (PPB) ranged from 86.36% (OPY-04) to a maximum of 100% (OPL-12 and OPO-19), with an average of 94.38 % polymorphism. Fig. 2 and Fig. 3 represent the extent of polymorphism observed among 60 accessions of *E. latifolia*as revealed by OPL-12 and OPO-19, respectively. The mean PIC value observed was 0.285 across all primers (Table 2).

The amplified RAPD bands were adequate to discriminate all accessions. The polymorphic bands were used to generate the similarity martrix. Jaccard's similarity coefficientor genetic similarity (GSj) ranged from 0.38-0.71. Dendrogram based on UPGMA analysis grouped the 60 accessions into main clusters.The 60 accessions from RAPD analysis were grouped into one main cluster and a single accession, H38, formed a separate out group showing a low similarity coefficient (0.38) with other accessions (Fig. 4). Accessions within main cluster were grouped into five groups. The first group I comprised L2, L42 and PY68. The group II consisted of 16 accessions, 6 accessions collected from Lamphun (L), 6 accessions collected from Lampang (LP) and 4 accessions from Mae Hong Son (MH). Group III was comprised of 21 accessions, 7 accessions collected from Nan (N), 9 accessions collected from Chiang Rai (CR) and 5 accessions collected from Phayao (PY). Within Nan, N55 and N72 appeared to be closer to each other, with the genetic similarity coefficient of 0.71. Their relatively high similarity may indicate that they developed from a common ancestor with similar fruit shape and taste. Group IV consisted of 13 accessions, 3 accessions collected from Mae Hong Son (MH) and 10 accessions collected from Chiang Mai (CM). The last group, group V comprised L59, MH61, MH63, LP59, CR29 and PY85. A cophenetic correlation tree of 60 accessions of *E. latifolia* was evaluatedusing seven primers. The cophenetic correlation of the similarity matrix (*r*) was 0.7202indicating that the cluster results had a good fit to the genetic similarity matrix.

Table 2. Total number of bands, number polymorphic bands, percentage of polymorphic bands and polymorphism information content (PIC) generated by 7 primers in 60 accessions of *Elaeagnuslatifolia*L. by RAPD analysis

Primer	Primer sequence (5′ - 3′)	Number of total band	Number of polymorphic band	% PPB	PIC
OPL12	GGG CGG TAC T	29	29	100	0.348
OPO19	GGT GCA CGT T	31	31	100	0.328
OPR04	CCC GTA GCA C	31	30	96.77	0.377
OPT12	GGG TGT GTA G	18	17	94.44	0.267
OPU13	GGC TGG TTC C	27	24	88.88	0.262
OPY04	GGC TGC AAT G	22	19	86.36	0.184
OPAJ18	GGC TGA GTG G	20	18	90.00	0.229
Total		178	168	94.38	0.285

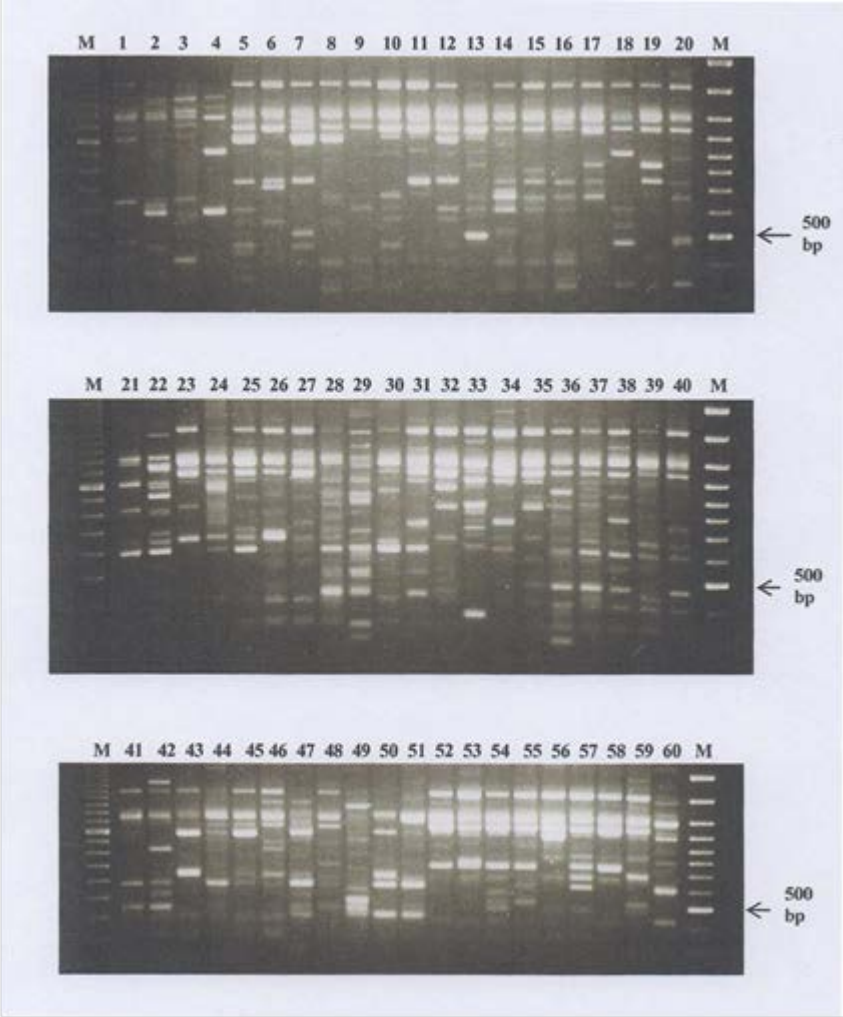

Fig.2. Banding patterns of 60 accessions of *Elaeagnuslatifolia* L. fromOPL-12 primer (M: 100 bp marker)

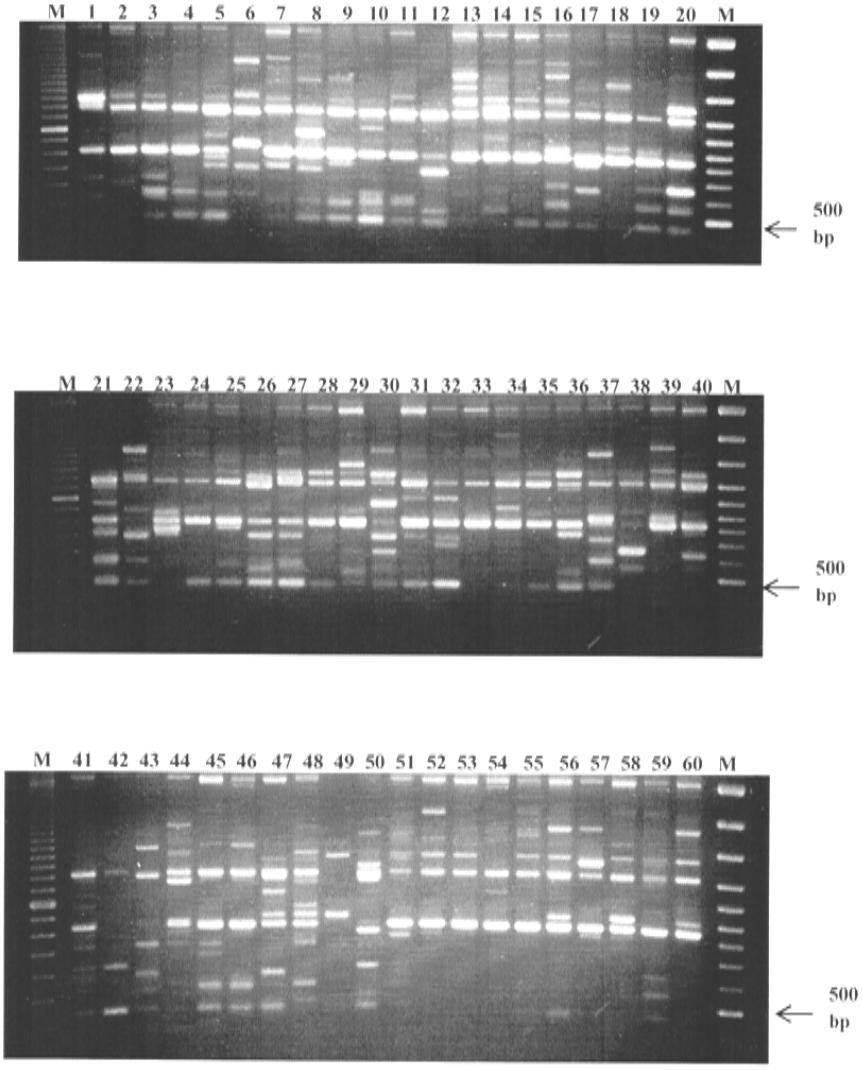

Fig.3. Banding patterns of 60 accessions of *Elaeagnuslatifolia* L. fromOPO-19 primer (M: 100 bp marker

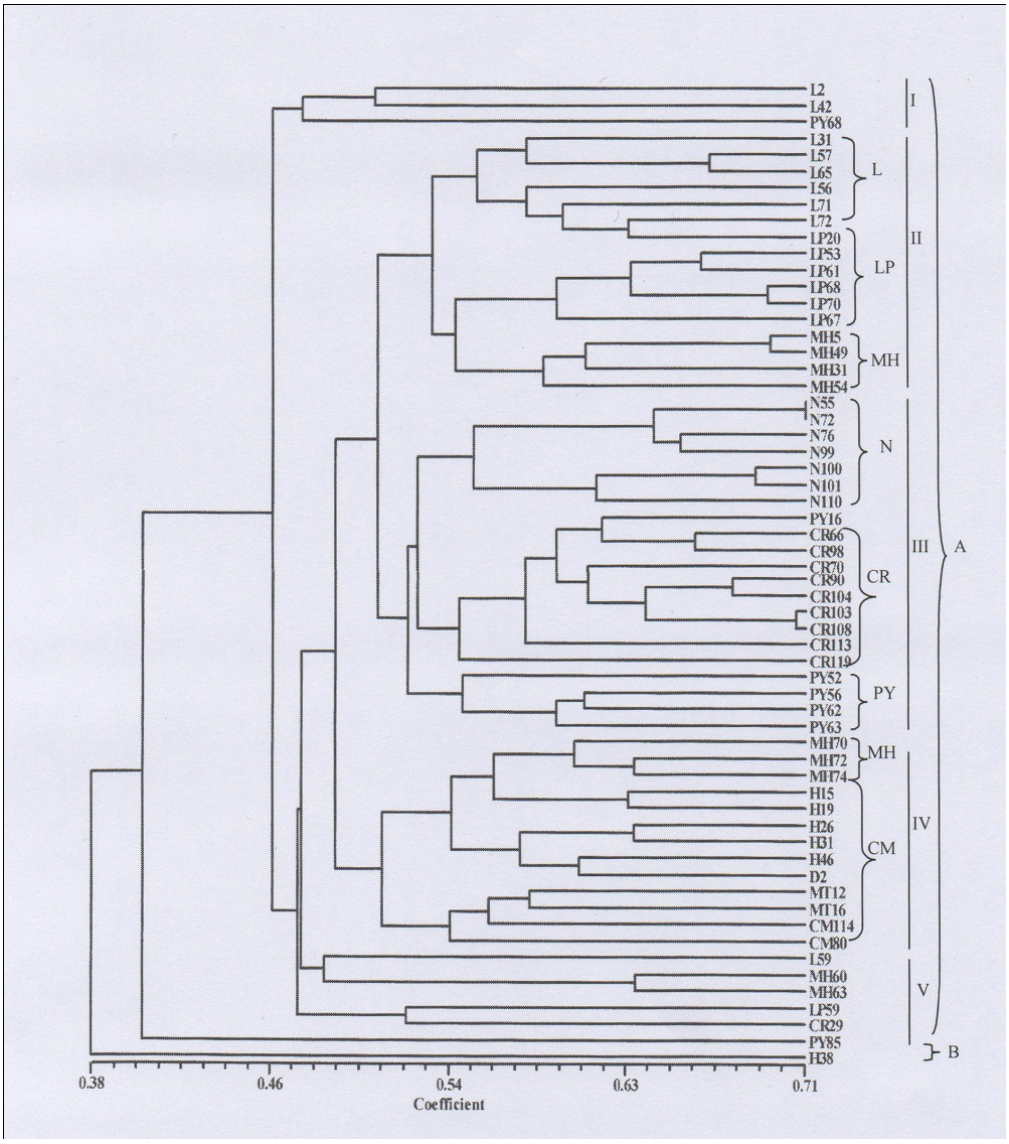

Fig.4. Dendrogram of 60 accessions of *Elaeagnuslatifolia* L. resulting from a UPGMA cluster analysis obtained from seven primers using Jaccard's similarity coefficient.

Discussions

Estimation of genetic diversity among and within crops is crucial in breeding and conservation of genetic resources. RAPD marker analysis is widely used for detection of genetic diversity and classification of the germplasm of many organisms. The different primers are used to amplify different numbers of genotypes. The number of genotypes or accessions

amplified by theses RAPD primers vary according to the primers used, which depend on the presence of amplification sites (Mahmood*et al.,* 2013). RAPDs have been shown to be an effective method for studying genetic diversity in many plants such as wheat (Al-Rawashdeh, 2011), *Jasminum*species (Mahmood*et al.,* 2013), mango,*Mangiferaindica* (Ramessur and Ranghoo-Sanmukhiya, 2011) and Chilli, *Capsicum annuum* (Peeraullee and Ranghoo-Sanmukhiya, 2013).

In this paper, RAPDs were used to estimate the genetic diversity of *E. latifolia*. Seven primers were selected for characterization of 60 accessions of *E. latifolia*. A total of 178 DNA fragments were identified, 168 were polymorphic. RAPD markers exhibited more than 60 % polymorphism indicating the presence of wide genetic variability among different genotypes and variations in DNA sequences leading to polymorphism. Therefore, this great polymorphism is indicative of great genetic diversity (Gajera *et al*., 2010). Jaccard's similarity coefficientor genetic similarity (GSj) ranged from 0.38-0.71. The results showed high level of polymorphism occurred in 60 accessions. Fifty eight accessions could be distinguished, while only two accessions; N55 and N72 appeared to be genetically very close with the genetic similarity coefficient of 0.71. Their relatively high similarity may indicate that they developed from a common ancestor with similar in leaf and fruit shapes as well as taste. Clustering patterns of different accessions in the dendrogram revealed that the genetic diversity of *E. latifolia* is not in perfect agreement with geographical diversity. The dendrogram showed that for some groups there was no clear pattern of clustering according to the locations from where accessions were collected. Al-Rawashdeh (2011) reported that genetic similarity was detected by RAPD analysis in the wild medicinal plant *Artemisia judaica*. High similarity indicators of the accessions in the current study suggest that the test entities have a close genetic relation. A slight correlation with geographic location was also noted which is similar to our study for the samples collected from the same location, however the different clusters are identified in the samples collected from the different areas. Same finding was reported by Tonk *et al*. (2011) on *Hypericum perforatum*, clustering based on RAPD data showed a moderate level of correlation with regional origin of the clones.

Conclusion

RAPD markers were found to be useful in the assessment of *E. latifolia*diversity. This understanding of genetic diversity can be used for genetic conservation and genetic resource management of this plant species in the future. Possible paths for future research on genetic diversity in populations

of *E. latifolia*is the use of advanced molecular markers techniques such as inter-simple sequence repeats (ISSRs) and/or simple sequence repeats (SSRs).

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