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Assessment of Genetic Diversity in *Elaeagnus Latifolia* L. by Inter-Simple Sequence Repeat (Issr) Markers

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Abstract Inter-simple sequence repeat (ISSR) markers were analyzed to study the genetic diversity and phylogenetic relationships among 88 accessions of *Elaeagnus latifolia* L. collected from the upper north of Thailand. Fourteen selected primers produced 278 discernible bands, with 264 (94.96%) being polymorphic, indicating high genetic diversity at the accession level. Jaccard's similarity coefficient or genetic similarity (GSj) ranged from 0.50-0.79. Eighty-six out of 88 accessions could be distinguished, whereas only two accessions. H31 and H38, appeared to be genetically close to each other, with a genetic similarity coefficient of 0.79. Our results indicated that the genetic relationships between accessions were related to collecting area, and a high level of genetic diversity was founded in *E. latifolia* populations. Therefore, ISSR markers can be used to differentiate closely related individuals and assess genetic diversity in *E. latifolia*.

Keywords: *Elaeagnus latifolia* L., DNA fingerprinting, Genetic diversity, ISSR markers

Introduction

Elaeagnus latifolia L., belongs to the Elaeagnaceae family. This species is distributed widely from the northern regions of Asia to the Himalayas and Europe (Ahmadiani *et al.*, 2000). *E. latifolia* is an endemic fruit plant mostly found in the upper north of Thailand (Yingthongchai *et al*., 2014). It is a large evergreen woody climber with rusty shiny scales that are often thorny. The climber covers the neighboring trees and it is difficult to estimate the length of the stems. The flowers are hermaphroditic and are pollinated by bees. Fruits are eaten raw or pickled. The fruit contains a single large seed (Petal *et al*., 2008; Sundriyal and Sundriyal, 2003). The fruit is nutritionally rich and can be utilized for making chutney, jam and jelly. The fruit yield per tree could easily compete with commercial fruit-yielding species. Therefore, such fruit trees

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deserve a high priority for conservation in natural forest stands and domestication in farmer's fields (Sundriyal and Sundriyal, 2003).

In the Sikkim Himalaya of India, *E. latifolia* is one of the species of wild fruit that is prized for both local consumption and its commercial potential (Sundriyal and Sundriyal, 2005; Patel *et al*., 2008). The species is found in the subtropical and temperate Himalayas from Kumaon through Sikkim, Darjeeling, Bhutan and the Khasi hills in Meghalaya. This fruit plant is a species that bears maximum pressure in natural habitats due to a high demand by the local population, and was studied in detail by (Sundriyal and Sundriyal, 2005; Patel *et al*., 2008). The native people of Mizoram, India use parts of *E. latifolia* in traditional medicine. For examples, the root juice (5-10 ml, 1-2 times daily) is given orally to remove retained placenta. A root decoction (10-15 ml, 3 times daily) is taken orally in the treatment of threatened abortion (Sharma *et al*., 2001). Therefore, the *E. latifolia* is an important plant because of its traditional and commercial value.

Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification (Awasthi *et al*., 2004), because phenotypic traits are highly influenced by environmental conditions (Ruan *et al*., 2009). Understanding of the genetic variation within and between populations is essential for the establishment of effective and efficient conservation for plants (Shafie *et al*., 2009).

Inter-simple sequence repeat (ISSR) markers is a relatively new molecular marker technique developed by Zietkiewicz *et al*. (1994), based on amplification of a single primer containing a microsatellite "core" sequence anchored at the 3′ or 5′ end by a set of 2-4 purine or pyrimidine residues. This technique is more specific, offers a high degree of reproducibility and a rich level of polymorphism in a relatively simple and low cost procedure, overcoming common criticism against the use of random amplified polymorphic DNA (RAPD). The use of inter-simple sequence repeats (ISSRs) is a microsatellite-derived genetic fingerprinting method based on the amplification of DNA segments occurring in the genome. They are advantageous because no prior genomic information is requiring for their use.

The technique is stable across a wide range of PCR parameters. This method is similar to RAPDs since both require no prior knowledge of the genome, cloning or specific primer design, it has higher reproducibility than RAPDs because of high annealing temperature, and the cost of the analyses is lower than the cost of amplified fragment length polymorphisms (AFLPs). ISSR-PCR uses a single primer composed of a di- or trinucleotide simple sequence repeat with or without a 5′- or 3′-anchoring sequence of 1-3

nucleotides (Ge *et al*., 2003). Currently, ISSR marker technique has been shown to be effective for DNA fingerprinting, genetic diversity analyses and germplasm evaluation. Hence it has been widely used in assessment of genetic diversity, clonal identity and cultivar identification (Bhattacharya *et al*., 2010) and ISSR amplification is a PCR-based method that can rapidly differentiate closely related individuals (Zietkiewicz *et al*., 1994).

ISSR markers have been used for genetic diversity analysis and cultivar identification in numerous plant species, e.g., *Hippophae* L. (Ruan *et al.*, 2009; Li *et al*., 2009), *Camellia sinensis* (Thomas *et al.*, 2006), *Jathopha* and *Jatropha*-related species (Thanya *et al*., 2011), *Olea europaea* (Terzopoulus *et al.*, 2005), *Citrus* spp. (Biswas *et al*., 2010), *Citrus indica* (Kumar *et al*., 2010), *Vanilla* species (Verma *et al*., 2009), *Dendrobium* species (Wang *et al*., 2009),), *Sorghum bicolor* ssp. *bicolor* (Tadesse and Feyissa, 2013) and *Punica granatum* (Narzary *et al*., 2009).

In the present study, ISSR markers were applied to evaluate genetic diversity and genetic relationship among 88 accessions of *E. latifolia* distributed in the upper North of Thailand.

Materials and methods

Plant materials

The 88 accessions of *E. latifolia*, were collected from eight provinces in the upper northern part of Thailand, i.e. Chiang Mai (CM), Chiang Rai (CR), Mae Hong Son (MH), Phrae (P), Nan (N), Phayao (PY), Lamphun (L) and Lampang (LP) (Figure 1 and Table 1) for ISSR analysis. Two to three young leaves were removed from each tree and stored in deep freezers (-20°C) until use.

DNA extraction

Total genomic DNA was extracted from approximately 100 mg young leaves using a DNeasy Plant Mini kit (Qiagen) following the protocol of the manufacturer. The quantity and quality of isolated total genomic DNA was determined relative to known concentrations using 0.8% agarose gels electrophoresis in 0.5X TBE buffer for mobility with EZ load Precision Molecular Mass Standard (Bio-Rad) and diluted to uniform concentration (10 ng/µl) for ISSR analysis.

DNA amplification

Fourteen of 100 ISSR primers producing clear and unambiguous bands were used to perform the PCR amplifications (UBC primer set No. 9, University of British Columbia, Canada). PCR amplifications were carried out in a Gradient Palm-Cycle™ thermocycler (The Corbett Research Life Science, Australia). Amplification reactions were carried out in 25 µl containing pure Taq Ready-To-Go PCR Beads (GE Healthcare UK Limited, UK), 0.2 µM of ISSR primer and 10 ng of template DNA. Amplification condition consisted of an initial step at 94 °C for 5 min, 40 cycles of 94 °C for 30 sec, 50 °C for 45 sec, 72 °C for 2 min, followed by final extension at 72 °C for 7 min.

Amplification products were electrophoresed for 2 h on 1.5 % agarose gels using 0.5 × TBE buffer at 120 V. A size marker, 100 bp plus Gene Ruller DNA (Fermentas), was used. After electrophoresis the gel was stained in ethidium bromide (0.5 µg/ml) and the patterns were photographed using a gel documentation system.

Data analysis

The bands observed 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 the unweighted pair-group method with an arithmetic average (UPGMA).

Genetic similarities between samples were measured by the Jaccard's similarity coefficient (GSj) using the 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.

Fig. 1. Characteristics of *Elaeagnus latifolia* L., A) scandent shrub B) fruits

Table 1. Eighty eight accessions of *Elaeagnus latifolia* L. from eight areas used in ISSR analysis

Area	Number of accessions	Location collected	Accessions code
Chiang Mai (CM)	29	Saraphi, Doisaket, Mae Taeng, Hot and San Pa Tong	HS1, HS2, HS3, HS6, HS12, HS13, SP1, SP2, SP3, SP4, SP6, MT12, MT16, MT17, SPT1, SPT2, SPT3, SPT4, D2, H11, H15, H19, H20, H26, H31, H38, H46, H48, H49 (No. 1-No. 29)
Lampang (LP)	7	Chae Hom, Mueang Pan and Mueang	LP20, LP53, LP59, LP61, LP67, LP68, LP70 (No. 30-No. 36)
Lumphun (L)	11	Pa Sang, Ban Hong, Li and Thung Hua Chang	L2, L31, L42, L56, L57, L59, L65, L71, L72, L74, L75 (No. 37-No. 47)
Phayao (PY)	10	Dok Kham Tai, Mueang, Mae Jai and Pu Kham Yoa	PY16, PY49, PY52, PY56, PY62, PY63, PY68, PY85, PY87, PY88 (No. 48- No. 57)
Chiang Rai (CR)	11	Mae Suai, Mae Chan, Mae Sai, Wiang Chai, Wiang Pa Pao and Chiang Saen	CR26, CR66, CR70, CR90, CR98, CR103, CR104, CR108, CR113, CR119, CR126 (No. 58- No. 68)
Phrae (P)	3	Wang Chin, Sung Men and Mueang Phrae	P3, P4, P6 (No. 69-No.71)
Nan (N)	8	Wiang Sa, Mueang, Pua, San Ti Suk and Bo Kluea	N55, N70, N72, N76, N99, N100, N101, N110 (No.72- No.79)
Mae Hong Son (MH)	9	Pai and Khun Yuam	MH5, MH31, MH49, MH54, MH60, MH63, MH70, MH72, MH74 (No. 80-No. 88)

Results

Amplified ISSR bands could be used to differentiate all accessions. ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs. The results of amplification of genomic DNA of 88 accessions, using 14 primers for ISSR analysis are indicated in Table 2. Fourteen primers (UBC807, UBC808, UBC809, UBC812, UBC-818, UBC825, UBC826, UBC827, UBC864, UBC881, UBC886, UBC888, UBC889 and UBC890) produced a very high percentage of polymorphic bands. Representative banding patterns observed with primers UBC864 and UBC886 are shown in Figure 2 and Figure 3. The number of fragments produced by each primer ranged from 10 (UBC-812) to 28 (UBC-886). A total of 278 DNA fragments were identified, of which 264 were polymorphic, with an average of 19.85 polymorphic fragments per primer. The percentage of polymorphic band (PPB) ranged from 90.40% (UBC-889) to a maximum of 100% (UBC-807, UBC-812 and UBC-827), with an average of 94.96 % polymorphism. The PPB suggested that ISSR markers were suitable to detect the genetic diversity of these accessions of *E. latifolia* at the DNA level.

A dendrogram based on UPGMA analysis with ISSR data is shown in Figure 4. Jaccard's similarity coefficient or genetic similarity (GSj) ranged from 0.50-0.79. The cophenetic correlation of the similarity matrix)*r*) was 0.7210 indicating that the cluster results had a good fit to the genetic similarity matrix. Eighty-six out of 88 accessions could be distinguished whereas only two accessions; H31 and H38 accessions appeared to be closer to each other, with the genetic similarity coefficient of 0.79. On the basis of the similarity dendrogram, the 88 accessions of *E. latifolia* could be classified into one main cluster and single accessions L72 formed a separate OUT group in the cluster, showing a lower similarity coefficient (0.50) with other accessions. Accessions within main cluster A were divided into four groups. Group I was comprised of 18 accessions collected from Chiang Mai (CM). Group II was comprised of 11 accessions collected from Chiang Mai (CM) and 7 accessions collected from Lampang (LP), within group II two accessions; H31 and H38 appeared to be closer to each other, with a genetic similarity coefficient of 0.79. Group III was comprised of 10 accessions collected from Lamphun (L), 10 accessions collected from Phayao (PY), 3 accessions collected from Phrae (P) and 11 accessions collected from Chiang Rai (CR) while group IV was comprised of 8 accessions collected from Nan (N) and 9 accessions collected from Mae Hong Son (MH). The results indicated that genetic relationships between populations related to collecting area and a high level of genetic diversity was found in *E. latifolia* populations.

Table 2. Total number of bands, number polymorphic bands, percentage ofpolymorphic bands (PPB) generated by 14 primers in 88 accessions of *Elaeagnus latifolia* L. by ISSR analysis

Primer	Primer sequence (5′ - 3′)	Number of total bands	Number of polymorphic bands	PPB
UBC-807	AGA GAG AGA GAG AGA GT	15	15	100.00
UBC-808	AGA GAG AGA GAG AGA GC	17	16	94.11
UBC-809	AGA GAG AGA GAG AGA GG	17	16	94.11
UBC-812	GAG AGA GAG AGA GAG AA	10	10	100.00
UBC-818	CAC ACA CAC ACA CAC AG	20	19	95.00
UBC-825	ACA CAC ACA CAC ACA CT	13	12	92.30
UBC-826	ACA CAC ACA CAC ACA CC	22	21	95.45
UBC-827	ACA CAC ACA CAC ACA CG	23	23	100.00
UBC-864	ATG ATG ATG ATG ATG ATG	26	24	92.30
UBC-881	GGG TGG GGT GGG GTG	16	15	93.75
UBC-886	VDV CTC TCT CTC TCT CT	28	26	92.85
UBC-888	BDB CAC ACA CAC ACA CA	24	23	95.83
UBC-889	DBD ACA CAC ACA CAC AC	22	20	90.90
UBC-890	VHV GTG TGT GTG TGT GT	25	24	96.00
Total		278	264	94.96

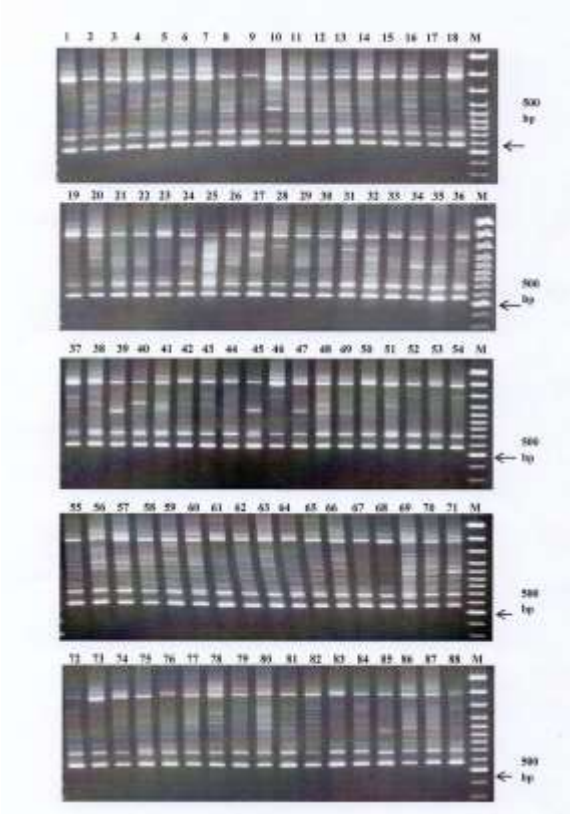

Fig. 2. Banding patterns of 88 accessions of *Elaeagnus latifolia* L.generated by ISSR using UBC-864 primer (M: 100 bp marker)

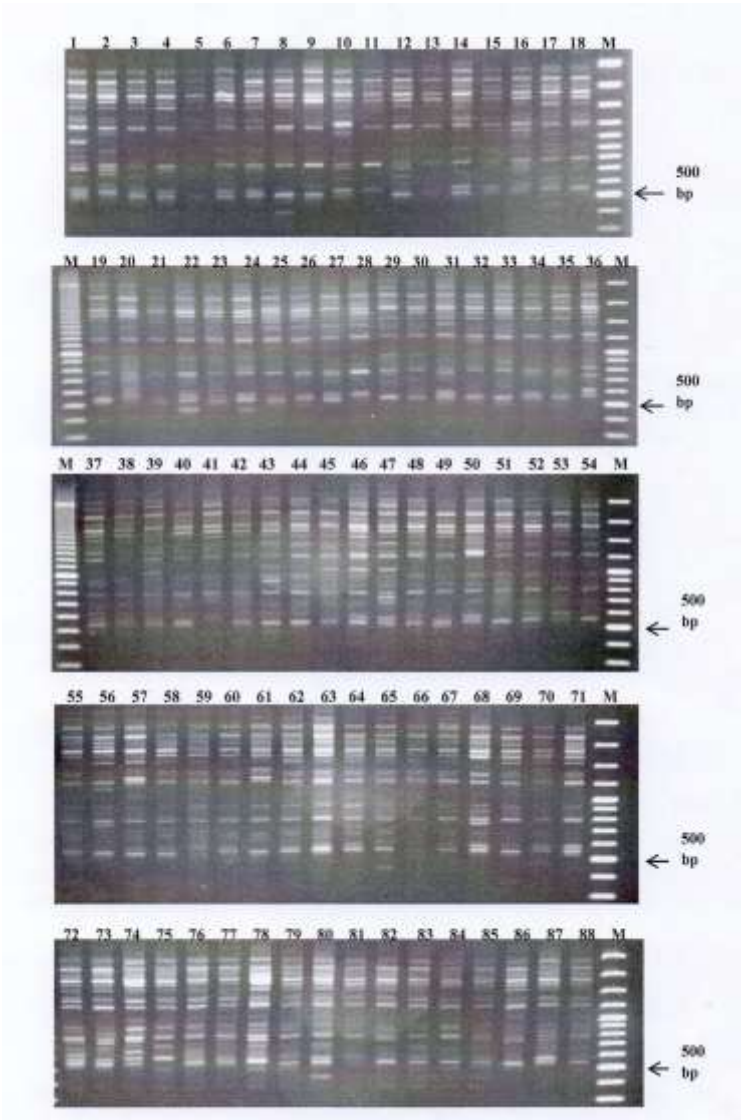

Fig. 3. Banding patterns of 88 accessions of *Elaeagnus latifolia* L. generated by ISSR using UBC-886 primer (M: 100 bp marker)

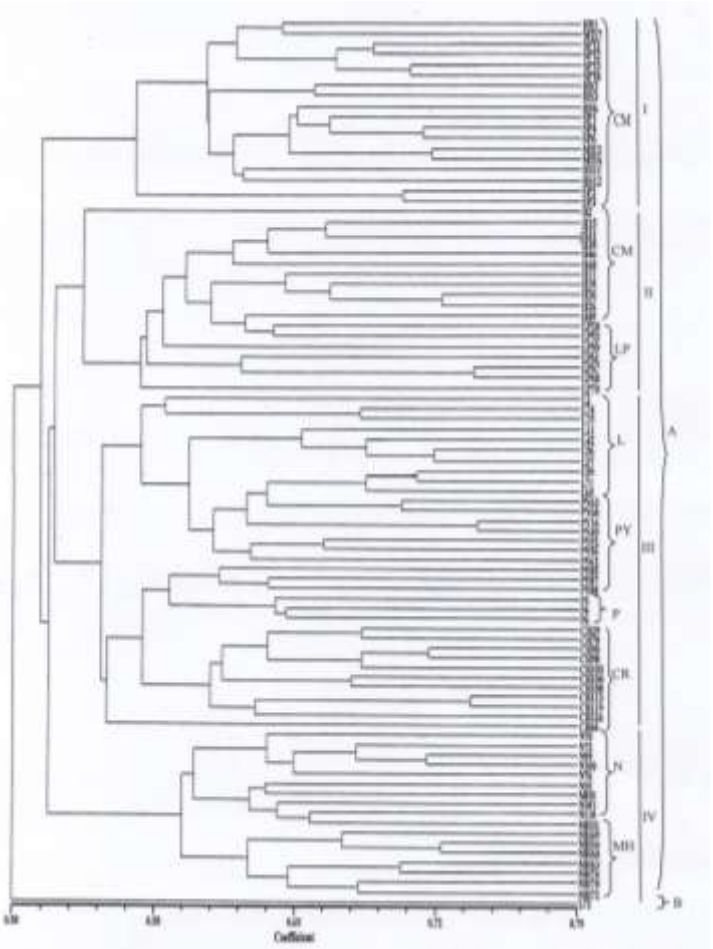

Fig. 4. Dendrogram of 88 accessions of *Elaeagnus latifolia* L. resulting from a UPGMA cluster analysis obtained using ISSR and 14 primers

Discussions

In the present study, ISSR markers were used to estimate the genetic diversity of *E. latifolia*. Many studies have reported that the ISSR technique is highly efficient and reproducible (Zhao *et al*., 2007). ISSR markers were useful in estimating genetic relations in *Physalis* from which the markers are also an effective tool for documenting interspecific variability, discriminating species, and assessing intraspecific similarity and differentiation (Vargas-Ponce *et al*., 2011). ISSR method was sufficiently informative and powerful to estimate the genetic diversity in *Murraya koenigii* (Verma and Rana, 2011).

In this study, 14 primers were selected for characterization of 88 accessions of *E. latifolia*. ISSR was successfully used to amplify plant DNA,

and polymorphism was found in all accessions. The fourteen primers used in this study indicated that a high level of polymorphism (94.96 % polymorphism) exists in the 88 accessions. The percentage of polymorphism ranged from 90.40% (UBC-889) to a maximum of 100% (UBC-807, UBC-812 and UBC-827). Primer UBC-807, UBC-812 and UBC-827 were most discriminatory in assessing genetic diversity between *E. latifolia* accessions. Vargas-Ponce *et al*. (2011) reported similar results; 6 ISSR primers produced 100% polymorphic bands, a very high interspecific percentage in *Physalis*. Verma and Rana (2011) reported that, 13 primers of UBC set gave a high percentage of polymorphism (89.47%) across all accessions of *Murraya koenigii.* Six primers of the UBC primer set were also used for characterization of citrus gerplasm including unknown variants, and indicated that ISSR markers are a powerful tool which can differentiate closely related individuals (Shahsavar *et al*., 2007).

Base on the Jaccard's similarity coefficient or genetic similarity (GSj) which ranged from 0.50-0.79, the 88 accessions could be easily distinguished whereas only two accessions; H31 and H38 appeared to be closely related, with the genetic similarity coefficient of 0.79.The genetic closeness of H31 and H38, may have been due to their close geographic proximity; these accessions were collected in the same village and they were similar in fruit color and taste. Thus, their relatively high similarity may indicate that they were developed from a common ancestor. Similarly, Chen *et al*. (2011) found that *Jatropha curcas* clones from the same place have a close genetic affinity and cluster together.

ISSR makers have been studied for assessment of genetic diversity in Iranian fennels (*Foeniculum vulgare* Mill.), an important medicinal plant. The results showed that ISSR markers provided a comprehensive insight into the genetic diversity of the ecotypes of Iranian fennel and efficiently identified 25 ecotypes of fennel thus allowing the characterization of the ecotypes under study (Bahmani *et al*., 2012). Similarly, ISSR markers clearly divided 88 accessions of *E. latifolia* into genetic relationship groups based on their geographical origin or collecting area.

Conclusion

The results of this study showed that ISSR markers can be used to differentiate closely related individuals and assess genetic diversity among all accessions of *E. latifolia* used in this study. Moreover, the information on genetic variability generated by our research could be useful for *E. latifolia* improvement programs.

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