Assessment of phylogenetic relationship among twenty *Curcuma* species in Thailand using amplified fragment length polymorphism marker

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**Abstract**

Plants in the genus *Curcuma* are a rhizomatous perennial herb which is widely distributed in Thailand. It has long been known for their uses as folk medicines, foods, spices, and cosmetics. However, the identification of plants in the genus *Curcuma* is very difficult due to morphological similarity in the early flowering stage. Recently, the molecular technique is one of the reliable and powerful tools for plant identification. In this study, the genetic relationship among twenty *Curcuma* species from Thailand was accessed by the amplified fragment length polymorphism (AFLP) method. AFLP fingerprint showed 98.54% highly polymorphisms with the number of bands (617 bands) ranging between 48 and 80 bands. The dendrogram generated from the unweighted pair group method of the arithmetic average could separate these *Curcuma* species into three major clusters. Cluster I can be subdivided into IA, which composed of *Curcuma parviflora*, *Curcuma sparganiifolia*, *Curcuma alismatifolia*, *Curcuma larsenii*, *Curcuma Gracillima*, and *Curcuma rhabdota* with similarity index (SI) 0.7926–0.9358 and IB composed of *Curcuma petiolata* and *Curcuma rubrobracteata* with the SI 0.9240. Cluster II can be subdivided into IIA being composed of *Curcuma longa*, *Curcuma Zedoaria*, and *Curcuma aromatica* with the SI 0.8989–0.9071, whereas Cluster IIB was composed of *Curcuma leucorrhiza*, *Curcuma aeruginosa*, *Curcuma comosa*, *Curcuma mangga*, *Curcuma angustifolia*, *Curcuma amada*, *Curcuma sessilis*, and *Curcuma albicoma* with the SI 0.8236–0.9500. Cluster III belongs to *Curcuma singularis* and *Alpinia galanga* (outgroup plant), which clearly separated into different clusters from twenty *Curcuma* species. In summary, the ten successful AFLP primer combinations could be used to determine the genetic relationship among closely related twenty *Curcuma* species in Thailand.

**Key words:** Amplified fragment length polymorphism, *Curcuma*, phylogenetic relationship

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**INTRODUCTION**

Plants in the genus *Curcuma* (family Zingiberaceae) comprise of >100 species widely distributed in Asia-tropical and Asia-Pacific regions. Several *Curcuma* species have long been known for their use as food, spices, cosmetics,
and ornamental plants. In addition, the Curcuma species was used in folk medicine for the treatment of diarrhea, dysentery, bronchial complaints, pneumonia, insect bites, and infectious wounds.\(^1,2\) The diarylheptanoids are the main active components in the rhizomes of Curcuma plants.\(^3\) The identification of Curcuma species has not yet been accomplished as there are some main problems in the taxonomic studies and lack of type specimens, etc.\(^4\) Moreover, in some cases, during the early flowering stage, the similarities in morphology led to confusion in their identification. Recently, several molecular techniques have been employed for taxonomic identification. Polymerase chain reaction (PCR) based on molecular markers was used to support the identification and distinguishing the genetic diversity analysis in medicinal plants such as the simple sequence repeat, random amplified polymorphic DNA (RAPD),\(^5\) and amplified fragment length polymorphism (AFLP) technique. Among this, AFLP not only has higher reproducibility and no prior sequence information but also has the capability to detect various polymorphisms in the genome simultaneously. As a result, this study is aimed to evaluate the phylogenetic relationships of twenty Curcuma species existing in Thailand, using the AFLP marker. The results might provide some useful information for studying the genetic relationship of the genus Curcuma.

**Plant materials**
Fresh rhizomes of twenty Curcuma species and Alpinia galanga (outgroup plant) were collected from various geographical areas in Thailand, as shown in Table 1. The shape and color of the rhizomes were recorded. All plant specimens were cultivated at the College of Public Health Sciences (CPHS), Chulalongkorn University, Thailand, for 1–2 months. Plant specimens were authenticated by Dr. Jenjittikul T, compared with the herbarium specimens at the Forest Herbarium Thailand, and then kept at CPHS.

**DNA extraction**
Genomic DNA was individually isolated from fresh young leaves (50–100 mg) using the modified cetyl trimethylammonium bromide (CTAB) method.\(^6\) The concentration and purity of genomic DNA were measured using a spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA) and then kept at −20°C for AFLP fingerprinting.

**Amplified fragment length polymorphism fingerprinting**
AFLP fingerprinting was performed as described by Vos et al., 1995, with some modification.\(^7\) Genomic DNA (100 ng/μL) was digested with EcoRI (5 U/μL) (Boehringer Mannheim GmbH, Germany) and Tvr9I (5 U/μL) (Roche Diagnostics GmbH, Germany) in buffer A (×10) (Promega, USA) at 37°C for 1 h. The ligation procedure was carried out at 37°C for 3 h to generate DNA template adapter. A subset of the restriction fragments was applied as a template in a preamplification reaction using MseI + C and EcoRI + A primers (Eurofins MWG Operon, Germany) and followed by selective amplification mixtures using three selective nucleotides of MseI and EcoRI primers performing in the PCR machine (Thermo Electron Corporation, USA). The selective amplification products of primer combinations were sizefractionated on denaturing polyacrylamide gel electrophoresis (6%) (Sigma, USA). Sliver was used to stain the AFLP fragments, and the AFLP fingerprint was evaluated.

**Data analysis**
The presence or absence of polymorphic AFLP bands was scored to generate a binary data set. The index of Jaccard was calculated for all pairwise comparisons in each species.\(^8\) The unweighted pair group method of the arithmetic average (UPGMA) dendrogram was constructed by Free Tree software (Pavlicek and Flegr, Prague, Czech Republic).\(^9\) Moreover, the bootstrap replication was computed using 1000 resampling subset of data.

**RESULTS**
Thirty-two AFLP primer combinations were screened. Ten primer combinations [Table 2] that showed a potentially high polymorphic band were accurately scored. The total of 617 bands ranged from 100 to 700 base pairs in size was

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**Table 1: List of twenty Curcuma species and Alpinia galanga**

| Scientific name         | Locality         |
|-------------------------|------------------|
| Curcuma aeruginosa Roxb. | Ratchaburi       |
| C. albicoma S.Q.Tong    | Chiang Mai       |
| C. alismatifolia Gagnep. | Prachin Buri     |
| C. amada Roxb.          | Chiang Mai       |
| C. angustifolia Roxb.   | Chiang Mai       |
| C. aromatic Salisb.     | Chiang Mai       |
| C. comosa Roxb.         | Chiang Mai       |
| C. gracilima Gagnep.    | Chiang Mai       |
| C. larsenii Makoi & Jenjitt. | Phetchabun   |
| C. leucorrhiza Roxb.    | Ratchaburi       |
| C. longa L.             | Chiang Mai       |
| C. mangga Valeton & Zijp | Chiang Mai     |
| C. parviflora Wall.     | Prachin Buri     |
| C. petiolata Roxb.      | Ratchaburi       |
| C. rhadbota Sirirugsa & M.F.Newman | Ubon Ratchathani |
| C. rubrobracteata Škorničk., M. Sabu & Prasanthk. | Chiang Mai |
| C. sessilis Gage        | Chiang Mai       |
| C. singularis Gagnep.   | Prachin Buri     |
| C. sparganitofila Gagnep. | Ubon Ratchathani |
| C. zedoaria (Christm.) Roscoe | Chiang Mai       |
| Alpinia galanga (L.) Wild. | Phetchabun     |

(Outgroup plants)
constructed, of which 608 bands (98.54%) were polymorphic and 9 bands (1.46%) were monomorphic bands. An average of AFLP banding was 61.7 bands by each primer combination, which were ranging from 48 to 80 bands. The total of 80 AFLP bands constructed from E + AAG/M + CAG primer combination showed the highest number, whereas 48 AFLP bands constructed from E + ACG/M + CTT primer combination showed the lowest number [Table 2]. The AFLP profile of twenty Curcuma and A. galanga (outgroup plant) obtained from E + AAG/M + CAG and E + AAC/M + CCA is shown in Figures 1 and 2.

For the genetic relationship analysis, UPGMA clustering using the Jaccard similarity matrix (SI) obtained from ten primer combinations is shown in Table 3. The similarity indices varied from 0.3165 to 0.9500. The clustering of phylogenetic relationships among the twenty Curcuma species and outgroup plants is shown in Figure 3. Three major clusters were classified as presented in the dendrogram. Cluster I can be divided into two subgroups, IA is composed of Curcuma parviflora, Curcuma sparganifolia, Curcuma alismatifolia, Curcuma larsenii, Curcuma gracillima, and Curcuma rhabdota with the SI 0.7926–0.9358, whereas Cluster IB being composed of Curcuma petiolata and Curcuma rubrobracteata with the SI 0.9240. Cluster II can be subdivided into IIA which composed of Curcuma longa, Curcuma zedoaria, and Curcuma aromatica with the SI 0.8989–0.9071, whereas Cluster IIB composed of Curcuma leucorrhiza, Curcuma comosa, Curcuma aeruginosa, Curcuma mangga, Curcuma angustifolia, Curcuma amada, Curcuma sessilis, and Curcuma albicoma with the SI 0.8236–0.9500. Cluster III belongs to Curcuma singularis, and Cluster IV belongs to A. galanga, outgroup plant, which clearly separated from twenty Curcuma species.

**DISCUSSION**

The taxonomic identification of the plant in the genus Curcuma is still unclear through the classical method based on the plant morphology, and some species descriptions are without Latin diagnosis or type specimen. In addition, the sample identification is quite difficult due to morphological similarity in early flowering, especially morphological
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Table 2: Ten primer combination and data obtained from amplified fragment length polymorphism analysis in twenty Curcuma species

| Primers combination | Primers sequence | Number of AFLP bands | Number of monomorphic bands | Number of monomorphic bands |
|---------------------|------------------|-----------------------|-----------------------------|-----------------------------|
| E+AAG/M+CAG         | 5’- AGA CTG CGT ACC AAT TCA AG ‑3’<br>5’- GAT GAG TCC TGA GTA ACA G ‑3’ | 80 | 1 | 98.75 |
| E+AGA/M+CGA         | 5’- AGA CTG CGT ACC AAT TCA GA ‑3’<br>5’- GAT GAG TCC TGA GTA ACA G ‑3’ | 74 | 0 | 100 |
| E+ACC/M+CAG         | 5’- AGA CTG CGT ACC AAT TCA CC ‑3’<br>5’- GAT GAG TCC TGA GTA ACA A ‑3’ | 70 | 1 | 98.57 |
| E+ACC/M+CCA         | 5’- AGA CTG CGT ACC AAT TCA CC ‑3’<br>5’- GAT GAG TCC TGA GTA ACA A ‑3’ | 65 | 2 | 96.92 |
| E+AGC/M+CTA         | 5’- AGA CTG CGT ACC AAT TCA GC ‑3’<br>5’- GAT GAG TCC TGA GTA ACT A ‑3’ | 60 | 0 | 100 |
| E+AGA/M+CCT         | 5’- AGA CTG CGT ACC AAT TCA GA ‑3’<br>5’- GAT GAG TCC TGA GTA ACT A ‑3’ | 60 | 1 | 98.33 |
| E+ACC/M+CCT         | 5’- AGA CTG CGT ACC AAT TCA AC ‑3’<br>5’- GAT GAG TCC TGA GTA ACT C ‑3’ | 54 | 1 | 98.15 |
| E+AAG/M+CCT         | 5’- AGA CTG CGT ACC AAT TCA AG ‑3’<br>5’- GAT GAG TCC TGA GTA ACA A ‑3’ | 54 | 1 | 98.15 |
| E+ACC/E+CAT         | 5’- AGA CTG CGT ACC AAT TCA AC ‑3’<br>5’- GAT GAG TCC TGA GTA ACA T ‑3’ | 52 | 2 | 96.15 |
| E+ACG/E+CTT         | 5’- AGA CTG CGT ACC AAT TCA GC ‑3’<br>5’- GAT GAG TCC TGA GTA ACT T ‑3’ | 48 | 0 | 100 |
| Total               |                  | 617                   | 9                           | 98.54 |

Figure 3: Unweighted pair group method of the arithmetic average dendrogram based on the index of Jaccard among twenty Curcuma species and Alpinia galanga

characteristics of rhizomes are highly similar. Sirirugsa et al. have been reported that using the presence/absence of stylodial glands and the shape of bract apex, Curcuma species in Thailand were divided into five groups, i.e., Alismatifolia, Cochinchinensis, Ecomata, Longa, and Petiolata groups. As a result, molecular markers which are reliable and powerful tools for identification are now commonly applied for the genetic diversity analysis in plants. The phylogenetic relationship among twenty Curcuma species was established using the AFLP marker. According to the constructed dendrogram, twenty Curcuma species can be divided into four clusters, and the similarity indices varied from 0.3165 to 0.9500 indicated the genetic diversity in the twenty Curcuma plants. The Jaccard similarity index (SI) was used to create
Table 3: Similarity index of twenty Curcuma species and Alpinia galangal similarity index value range from 0 to 1.0000 according to the increasing similarity index

|       | C. longa | C. zedoaria | C. aeruginosa | C. mangga | C. angustifolia | C. sessilis | C. petiolata | C. aromatic | C. rubrobracteata | C. albicoma |
|-------|----------|-------------|---------------|-----------|----------------|-------------|--------------|-------------|------------------|------------|
| C. longa | 1.0000   |             |               |           |                |             |              |             |                  |            |
| C. zedoaria | 0.9002   | 1.0000      |               |           |                |             |              |             |                  |            |
| C. aeruginosa | 0.8117   | 0.7621      | 1.0000        |           |                |             |              |             |                  |            |
| C. mangga | 0.8297   | 0.7876      | 0.8430        | 1.0000    |                |             |              |             |                  |            |
| C. angustifolia | 0.8451   | 0.7956      | 0.8398        | 0.8884    | 1.0000         |             |              |             |                  |            |
| C. sessilis | 0.8211   | 0.7606      | 0.8079        | 0.8527    | 0.9500         | 1.0000      |              |             |                  |            |
| C. petiolata | 0.8382   | 0.8078      | 0.8043        | 0.8182    | 0.8259         | 0.7888      | 1.0000       |             |                  |            |
| C. aromatic | 0.8989   | 0.9071      | 0.7449        | 0.7742    | 0.7787         | 0.7576      | 0.7739       | 1.0000      |                  |            |
| C. rubrobracteata | 0.8191   | 0.7922      | 0.7852        | 0.7992    | 0.8070         | 0.7767      | 0.9240       | 0.7583      | 1.0000           |            |
| C. albicoma | 0.8310   | 0.7851      | 0.8330        | 0.8857    | 0.9099         | 0.8698      | 0.8194       | 0.7682      | 0.8039           | 1.0000     |
| C. amada | 0.7843   | 0.7419      | 0.7925        | 0.8375    | 0.8613         | 0.8249      | 0.7813       | 0.7282      | 0.7691           | 0.9447     |
| C. comosa | 0.8620   | 0.8020      | 0.8421        | 0.8785    | 0.8753         | 0.8330      | 0.8459       | 0.7924      | 0.8375           | 0.8725     |
| C. parviflora | 0.8039  | 0.7665      | 0.7700        | 0.7981    | 0.8093         | 0.7754      | 0.8527       | 0.7500      | 0.8408           | 0.7992     |
| C. larsenii | 0.8004   | 0.7631      | 0.7700        | 0.8016    | 0.7988         | 0.7685      | 0.8636       | 0.7331      | 0.8516           | 0.7922     |
| C. leucorrhiza | 0.8635   | 0.8176      | 0.8586        | 0.9112    | 0.9115         | 0.8683      | 0.8401       | 0.8008      | 0.8317           | 0.9050     |
| C. habdota | 0.7868   | 0.7563      | 0.7632        | 0.7845    | 0.7922         | 0.7617      | 0.8499       | 0.7296      | 0.8236           | 0.7891     |
| C. sparganiifolia | 0.7832   | 0.7490      | 0.7422        | 0.7636    | 0.7748         | 0.7510      | 0.8395       | 0.7188      | 0.8203           | 0.7646     |
| C. alismatifolia | 0.7786   | 0.7515      | 0.7481        | 0.7763    | 0.7806         | 0.7500      | 0.8382       | 0.7213      | 0.8191           | 0.7773     |
| C. singularis  | 0.7456   | 0.7112      | 0.7351        | 0.7709    | 0.7510         | 0.7267      | 0.7845       | 0.6943      | 0.7863           | 0.7475     |
| C. gracilima | 0.7845   | 0.7437      | 0.7608        | 0.7786    | 0.7899         | 0.7697      | 0.8477       | 0.7271      | 0.8320           | 0.7797     |
| A. galanga | 0.3224   | 0.3227      | 0.3312        | 0.3299    | 0.3299         | 0.3165      | 0.3638       | 0.3168      | 0.3614           | 0.3292     |

Contd.....
Table 3: Contd.....

|     | C. amada | C. comosa | C. parviflora | C. larsenii | C. leucorrhiza | C. rhabdota | C. sparganiifolia | C. alismatifolia | C. singularis | C. gracillima | C. albicoma | C. longa | C. zedoaria | C. aeruginosa | C. mangga | C. angustifolia | C. sessils | C. petiolata | C. aromatica | C. rubrobracteata | C. alismatifolia | C. singularis | C. gracillima | A. galanga |
|-----|----------|-----------|---------------|-------------|----------------|-------------|-------------------|-----------------|--------------|--------------|------------|---------|-------------|--------------|----------|----------------|-----------|------------|--------------|----------------|----------------|-------------|--------------|-----------|
| C. amada | 1.0000   |           |               |             |                |             |                   |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. comosa | 0.8256   | 1.0000    |               |             |                |             |                   |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. parviflora | 0.7642   | 0.8330    | 1.0000        |             |                |             |                   |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. larsenii | 0.7539   | 0.8189    | 0.9030        | 1.0000      |                |             |                   |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. leucorrhiza | 0.8571   | 0.8974    | 0.8236        | 0.8272      | 1.0000        |             |                   |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. rhabdota | 0.7539   | 0.8089    | 0.8927        | 0.9358      | 0.8136        | 1.0000      |                   |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. sparganiifolia | 0.7396   | 0.7915    | 0.8707        | 0.9132      | 0.7926        | 0.8910      | 1.0000             |                 |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. alismatifolia | 0.7387   | 0.7973    | 0.8768        | 0.9076      | 0.8055        | 0.9089      | 0.8825             | 1.0000          |              |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. singularis | 0.7220   | 0.7819    | 0.7676        | 0.7607      | 0.7656        | 0.7539      | 0.7466             | 0.7422          | 1.0000       |              |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| C. gracillima | 0.7446   | 0.8101    | 0.8790        | 0.9177      | 0.8043        | 0.9072      | 0.8848             | 0.8949          | 0.7481       | 1.0000       |            |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
| A. galanga | 0.3240   | 0.3422    | 0.3463        | 0.3519      | 0.3279        | 0.3542      | 0.3354             | 0.3501          | 0.3362       | 0.3451       | 1.0000     |         |             |              |          |                 |          |            |              |                 |                 |             |              |           |
the dendrogram (bootstrap $P > 70\%$). High bootstrapping values demonstrated that each branch in the dendrogram was robust and stable. Molecular markers have been widely used as a tool for taxonomic and phylogeny diversity in *Curcuma* plants. Previous studies on the genetic variables in genus *Curcuma*, including *C. longa* by various molecular markers,[11-16] also demonstrated high polymorphism between and within this genus, which corroborating with the recent study. The UPGMA dendrograms based on the AFLP profile in this study revealed that twenty *Curcuma* species were divided into three major clusters. Subcluster IA composed of *C. parviflora*, *C. sparganiifolia*, *C. alismatifolia*, *C. larsenii*, *C. gracillima*, and *C. rhadbota* with the SI 0.9226–0.9358. According to their phenotype, this subgroup was considered as the *Alismatifolia* group that has the coma bract and bract and obtuse to rounded or acute bract apex, whereas subcluster IB composed of *C. petiolata* and *C. rubrobracteata* with the SI 0.9240 was considered as the *Petiolata* group that has the straight acicular anther spurs, clavate stylodial gland, and coma bract with rounded to obtuse bract apex. Cluster II can be subdivided into IIA which composed of *C. longa*, *C. zedoaria*, and *C. aromaticum* with the SI 0.8989–0.9071, whereas Cluster IIB composed of *C. leucorrhiza*, *C. conosoma*, *C. aeruginosa*, *C. mangga*, *C. angustifolia*, *C. amada*, *C. sessilis*, and *C. albicoma* with the SI 0.8236–0.9500. *Curcuma* species in cluster II was considered as the *Longa* group, which has the curved acicular anther spurs and cylindrical stylodial gland along with coma bract with acute bract apex. Cluster III belongs to *C. singularis* and *A. galanga* (outgroup plants) is clearly separated from twenty *Curcuma* species in a different branch of the tree with 100% bootstrap data in the UPGMA dendrogram. The result was in agreement with previously reported in 15 *Curcuma* species from Thailand, using RAPD marker, and the result found that 15 *Curcuma* species can be divided into three major groups with the SI ranged from 0.0909 to 0.9222.[17] Based on RAPD and ISSR marker, *C. longa* and *C. zedoaria* were grouping in the same cluster, whereas based on PCR-restriction fragment length polymorphism, *C. aromaticum* and *C. zedoaria* were grouping in the same cluster.[11,18,19] In addition, Cao et al. and Zaveska et al. reported that based on ITS, trnK, and chloroplast DNA sequences, *C. rubrobracteata* was clustered with *C. petiolata*, whereas *C. longa* was clustered with *C. zedoaria*. A wide range of pairwise genetic distances indicated the genetic diversity. The chloroplast genome sequences have been also utilized for studying sequence variation in the plant such as *Maturase K*, which is a promising DNA barcode of Zingiberaceae, including *Curcuma* species but not for *C. longa* due to the conservation of *matK* gene within this species.[22] The identification of *Curcuma* species has been achieved using morphological data by many researchers. However, confusion still persists due to the *Curcuma* species exhibited large morphological variations. As a result, the present investigation demonstrates the efficiency and reliability of the AFLP marker for the determination of genetic diversity among twenty *Curcuma* species existing in Thailand.

**CONCLUSION**

Ten successful AFLP primer combinations could be applied for determining genetic relationships among closely related twenty *Curcuma* species in Thailand. This research revealed that the phylogenetic relationships of twenty *Curcuma* species were correlated with the morphological characteristics.

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**Conflicts of interest**

There are no conflicts of interest.

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