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Genetic relationships among five varieties of *Curcuma alismatifolia* (Zingiberaceae) based on ISSR markers

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ABSTRACT. The genus *Curcuma* is a member of the ginger family (Zingiberaceae) that has recently become popular for use as flowering pot plants, both indoors and as patio and landscape plants. We used PCR-based molecular markers (ISSRs) to assess genetic variation and relationships between five varieties of curcuma (*Curcuma alismatifolia*) cultivated in Malaysia. Sixteen ISSR primers generated 139 amplified fragments, of which 77% had high polymorphism among these varieties. These markers were used to estimate genetic similarity among the varieties using Jaccard's similarity coefficient. The similarity matrix was used to construct a dendrogram, and a principal component plot was developed to examine genetic relationships among varieties. Similarity coefficient values ranged from 0.40 to 0.58 (with a mean of 0.5) among the five varieties. The mean value of number of observed alleles, number of effective alleles, mean Nei's gene diversity, and Shannon's information index were 8.69, 1.48, 0.29, and 0.43, respectively.

Key words: *Curcuma alismatifolia*; Genetic relationship; ISSR-PCR; Molecular identification

INTRODUCTION

Curcuma alismatifolia is a monocotyledonous perennial, a member of the ginger family (Zingiberaceae) originating from tropical and subtropical areas of northern Thailand and Cambodia (Apavatjrut et al., 1999). *Curcuma* species have colorful, long-lasting inflorescences with few pest problems. They are used as cut flowers, flowering pot plants and as a garden plant (Roh and Lawson, 1993), and the plants comprise a number of pink coma bracts in the upper part and green coma bracts in the lower part, with small true flowers (Hagiladi et al., 1997). A well-known species from ancient times is *Curcuma longa*, turmeric, which is a major spice as well as the major source of yellow dye for coloring food and cloth in most South and Southeast Asian countries (Perry, 1980). Recently *C. alismatifolia*, a species native to Indo-China (Khuankaew et al., 2010), has gained popularity in the international market as a new ornamental bulb with a beautiful inflorescence. DNA-based molecular markers show differences in nucleotide sequences of DNA, which are now well established as powerful and versatile tools in the fields of plant breeding, taxonomy, physiology, genetic engineering (Kesawat and Das Kumar, 2009) genome mapping, and gene tagging (Bornet and Branchard, 2001, 2004). The inter-simple sequence repeat (ISSR)-PCR technique was chosen for assessing genetic variation in this study. ISSRs are randomly distributed throughout the genome and use simple sequence repeats anchored at the 5'- or 3'-end by a short arbitrary sequence as PCR primers (Zietkiewicz et al., 1994). They provide a powerful tool for genetic mapping and assessment of genetic diversity between closely related species and to detect similarities between and within species as well (Davilá et al., 1998; Moreno et al., 1998; Ghariani et al., 2003). The ISSR technique has been reported as a good alternative to AFLP when tested on *Curcuma* species (Syamkumar and Sasikumar, 2007; Das et al., 2011), roses (Jabbarzadeh et al., 2010), and pea and strawberry varieties, where it is less expensive, more rapid and more reproducible. The main advantage of ISSRs is that no sequence data for primer construction are needed. Since the analytical procedures include PCR, only low quantities of template DNA are required. Although, molecular markers have already been used either alone (Das et al., 2011) or together with agro-morphological traits (Syamkumar and Sasikumar, 2007) to assess the genetic diversity in other *Curcuma* species, there are no reports on the use of ISSR markers to analyze the relationship between *C. alismatifolia* varieties. The objectives of the present study were to determine genetic relationships and identify molecular fingerprints in five *C. alismatifolia* varieties using ISSR markers.

MATERIAL AND METHODS

Source of plant materials

The study was conducted at the Floriculture Laboratory, Crop Science Department, Faculty of Agriculture, University Putra Malaysia. Five *C. alismatifolia* varieties, namely Chiang Mai Pink, Sweet Pink, Doi Tung 554, Chiang Mai Red, and Kimono Pink, were purchased from Curcuma Nursery (Ubonrat), in Doisaket District, Chiang Mai 50220, Thailand.

DNA extraction

Fully opened fresh tender leaves of the *Curcuma* varieties were used for the extraction of DNA. Genomic DNA was isolated by the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The extraction buffer contained 2% (w/v) CTAB, 1.4 mM NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% (w/v) PVP and 2% (v/v) β-mercaptoethanol. The concentration and purity of the isolated DNA was determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc.) and the quality verified by electrophoresis on a 0.8% agarose gel.

PCR conditions

The ISSR reaction was carried out in a 25-µL reaction volume containing 1 µL genomic DNA, 2X DreamTaq™ Green PCR Master Mix (Fermentas International, Inc.) with 1 µM oligodeoxynucleotide primer. Amplification was performed in a thermal cycler (Bio-Rad Laboratories, Inc.) for total of 40 cycles after an initial denaturation of the template DNA at 94°C for 3 min. Initial denaturation was followed by 10 cycles of 94°C for 40 s, touch-down one-degree decrease in annealing temperature starting at 7°C above Tm for each primer for 30 s and 72°C for 1 min. This was followed by 30 cycles of 95°C for 40 s, last annealing temperature for 30 s (Table 1) and 72°C for 1 min and a final extension of 72°C for 10 min. The amplification products were analyzed on a 2% agarose gel with a 100-bp DNA ladder (N3231S, Biolabs, Inc.). The gel was stained by ethidium bromide, visualized under ultraviolet light and photographed using a gel documentation system (ChemilImager™ Gel Doc., Alpha Innotech Corporation, CA, USA).

Data scoring and analysis

Only clear, unambiguous and reproducible bands were considered for data analysis. Each band was considered to be a single locus. Data were scored as ‘‘1’’ for presence and ‘‘0’’ for absence. The binary data matrix was entered into the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc 2.10e; Rohlf, 2002). The level of similarity between species was established as the percentage of polymorphic bands, and a matrix of genetic similarity was compiled using Jaccard’s similarity coefficient (JSI) (Jaccard, 1908). Similarity coefficients were used to construct the dendrogram using the unweighted pair group method with arithmetic average (UPGMA) and the sequential hierarchical and nested clustering routine in the NTSYS-pc program, representing genetic relationship between five *Curcuma* varieties. Measurement of diversity including Nei’s gene diversity (h), observed number of alleles (N_A), effective number of alleles (N_E), Shannon information index (I) and the percentage of polymorphic bands (PPBs) were estimated by the POPGENE 1.31 software (Yeh et al., 1997).

RESULTS AND DISCUSSION

ISSR analysis

Of 18 ISSR primers, 16 exhibited polymorphism with the five *Curcuma* varieties (Figure 1) and are summarized in Table 1.

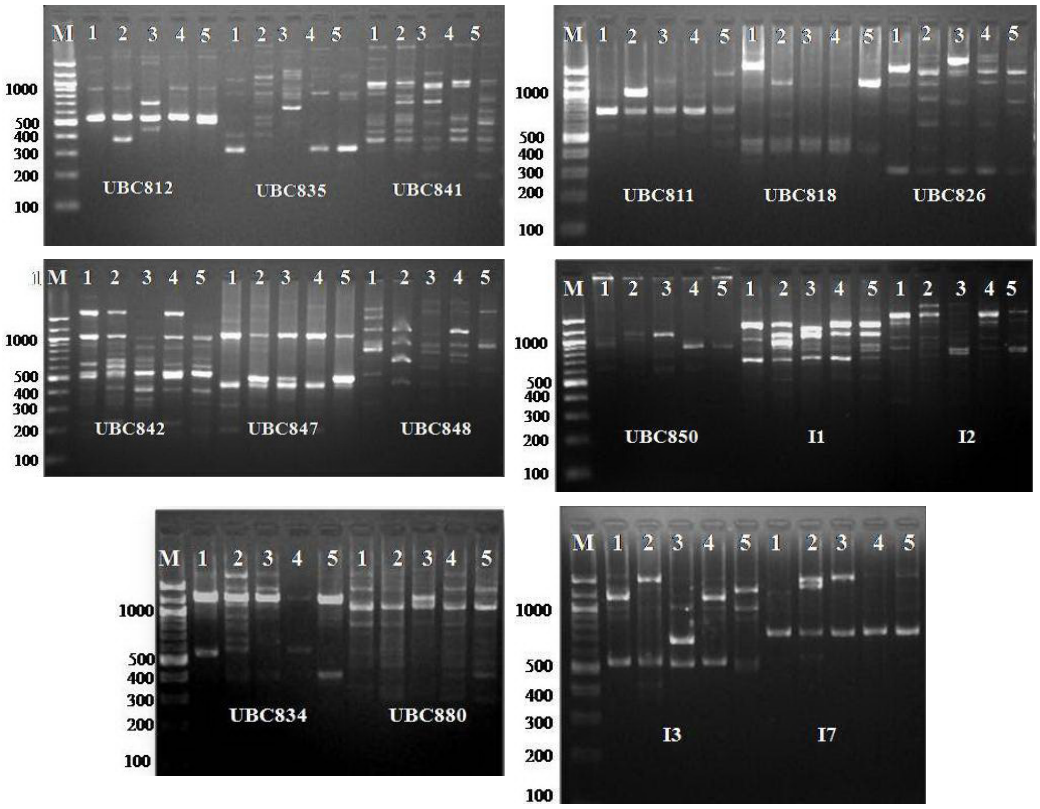

Figure 1. PCR products of genomic DNA from 5 *Curcuma alismatifolia* varieties with 16 ISSR primers. *Lane M* = 100-bp DNA marker; *lane 1* = Chiang Mai Pink; *lane 2* = Sweet Pink; *lane 3* = Doi Tung 554; *lane 4* = Chiang Mai Red; *lane 5* = Kimoni Pink.

Table 1. List of ISSR primers and its sequence used for the analysis.

Serial number	Primer	Sequence (5'-3')	Tm	Ta
1	UBC_811	GAGAGAGAGAGAGAGAC	57.2	64-54
2	UBC_812	$(GA)_8$A	54.8	62-52
3	UBC_818	CACACACACACACACAG	57.2	64-54
4	UBC_826	ACACACACACACACACC	57.2	64-54
5	UBC_834	$(AG)_8$YT	56.5	64-54
6	UBC_835	AGAGAGAGAGAGAGAGYC	58.8	66-56
7	UBC_841	GAGAGAGAGAGAGAGAY	58.8	66-56
8	UBC_842	GAGAGAGAGAGAGAGAYG	58.8	66-56
9	UBC_847	CACACACACACACACARC	58.8	66-56
10	UBC_848	CACACACACACACACARG	58.8	66-56
11	UBC_850	GTGTGTGTGTGTGTGTYC	58.8	66-56
12	UBC_880	GGAGAGGAGAGGAGA	56.2	63-53
13	I1	$(GA)_9$C	48.5	56-46
14	I2	$(GA)_9$T	48.0	55-45
15	I3	$(GA)_9$A	49.3	56-46
16	I7	$(CT)_8$G	44.9	52-42

Y = (C, T); R = (A, G). Tm = melting temperature (°C). Ta = anneling temperature (°C).

Sixteen ISSR primers detected a total of 139 amplification fragments, varying from 3 (I7) to 18 (UBC-841) fragments per primer and ranging from 180 to 2151 bp in size (Table 2). All primers tested revealed polymorphisms between the five varieties ranging from 25% for primer UBC-848 to 100% for primer UBC-850. The overall polymorphism (PPB) for the 16 primers across all five varieties was 77% (Table 2), suggesting that ISSR markers were polymorphic markers suitable to detect the genetic diversity of these varieties of *Curcuma* at the DNA level. The level of genetic diversity in the varieties of *C. alismatifolia* in our study ($I = 0.4324$) was lower than that reported in previous studies using ISSR ($I = 0.51 \pm 0.17$) and RAPD ($I = 0.53 \pm 0.19$). However, the average I in the present study was higher than that reported for AFLP markers ($I = 0.38 \pm 0.19$) indicating that a relatively great genetic diversity lies in the varieties of *Curcuma* (Das et al., 2011).

Table 2. ISSR polymorphic primer sequences used for analysis of the 5 varieties of *Curcuma alismatifolia* with number of bands amplified, number of polymorphic bands amplified, percentage of polymorphism, product size, number of effective alleles, Nei's (1973) gene diversity, and Shannon's index.

Locus	Total No. of bands	No. of polymorphic bands	PPB (%)	Product size (bp)	N_A	N_E	H	I
UBC_811	10	8	80.00	1517-540	1.8000 (0.4216)	1.4200 (0.2633)	0.2720 (0.1518)	0.4176 (0.2266)
UBC_812	8	5	62.50	1570-348	1.6250 (0.1575)	1.2941 (0.2436)	0.2000 (0.1656)	0.3128 (0.2590)
UBC_818	6	5	83.33	1781-420	1.8333 (0.4082)	1.4676 (0.2919)	0.2933 (0.1573)	0.4458 (0.2290)
UBC_826	10	9	90.00	2019-300	1.9000 (0.3162)	1.6045 (0.3093)	0.3520 (0.1470)	0.5194 (0.2017)
UBC_834	11	10	90.91	1810-413	1.9091 (0.3015)	1.6335 (0.3088)	0.3636 (0.1447)	0.5334 (0.1968)
UBC_835	13	11	84.62	1860-295	1.8462 (0.3755)	1.5722 (0.3332)	0.3323 (0.1660)	0.4898 (0.2324)
UBC_841	18	15	83.33	1860-180	1.8333 (0.3835)	1.5178 (0.3113)	0.3111 (0.1597)	0.4649 (0.2272)
UBC_842	8	3	37.50	2151-234	1.3750 (0.5175)	1.2330 (0.3506)	0.1400 (0.1994)	0.2092 (0.2936)
UBC_847	4	2	50.00	1176-196	1.5000 (0.5774)	1.3484 (0.4427)	0.200 (0.2400)	0.2934 (0.3460)
UBC_848	4	1	25.00	1860-500	1.2500 (0.5000)	1.1176 (0.2353)	0.0800 (0.1600)	0.1251 (0.2502)
UBC_850	6	6	100.0	1139-592	2.0000 (0.0000)	1.6968 (0.2478)	0.4000 (0.0876)	0.5867 (0.0945)
UBC_880	13	10	76.92	1907-300	1.8462 (0.5547)	1.5951 (0.3863)	0.3292 (0.1988)	0.5026 (0.3128)
I1	9	7	77.78	1329-539	1.7778 (0.4410)	1.4666 (0.3264)	0.2844 (0.1749)	0.4276 (0.2531)
I2	8	6	75.00	1863-375	1.7500 (0.4629)	1.4661 (0.3489)	0.2800 (0.1864)	0.4185 (0.2690)
I3	8	7	87.5	1557-528	1.8750 (0.3536)	1.5814 (0.3246)	0.3400 (0.1586)	0.5026 (0.2203)
I7	3	2	66.67	1537-742	1.6667 (0.5774)	1.4646 (0.4616)	0.2667 (0.2444)	0.3911 (0.3496)
Total	139	107	77					
Mean	8.69	6.69			1.4860 (0.3279)	1.4860 (0.3279)	0.2901 (0.1729)	0.4324 (0.2482)

N_A = observed number of alleles; PPB (%) = percentage of polymorphic bands; N_E = effective number of alleles; H = Nei gene diversity; I = Shannon index.

As a whole, the selected ISSR primers generated an average of 8.69 alleles and 1.48 effective alleles per locus. On a per locus basis, these numbers were much higher than the average of 1-6 alleles per locus for various ISSR loci reported by Das et al. (2011). The average number of alleles per locus obtained in the present study was also higher than that reported in previous studies using other types of markers such as isozymes (Paisooksantivatana et al., 2001). However, the numbers obtained in the present study were smaller than those reported by Syamkumar and Sasikumar (2007) who observed an average of 11.3 alleles per locus over eight ISSR loci. The estimates of genetic similarity ranged from 40% for the most distant varieties (Doi Tung 554 and Chiang Mai Pink) to 58% between the Chiang Mai Red variety and Kimono Pink (Table 3).

Table 3. Genetic similarity indices between each pair of the 5 curcuma varieties (*Curcuma alismatifolia*) based on ISSR fragment analysis.

Varieties	Chiang mai pink	Sweet pink	Doi tung 554	Chiang mai red
Sweet Pink	0.45			
Doi Tung 554	0.40	0.45		
Chiang Mai Red	0.48	0.51	0.44	
Kimono Pink	0.46	0.48	0.49	0.58

The UPGMA dendrogram (Figure 2) showed two main clusters. The first one included only the Doi Tung 554 variety and showed JSI of 0.45 with the other varieties, while the second included two groups. Chiang Mai Red and Kimono Pink showed maximum similarity between them (JSI = 0.58), forming the first group in cluster II. Sweet Pink variety had a JSI = 0.5 with Chiang Mai Red and Kimono Pink. The lone variety Chiang Mai Pink showed a JSI = 0.465 with Sweet Pink, Chiang Mai Red, and Kimono Pink, forming the second group in cluster II. Although there are published reports on the use of other molecular marker techniques such as RAPD, AFLP and ISSR to analyze various species of *Curcuma* in the past, this is the first report of the use of ISSR markers in analyzing *C. alismatifolia* varieties. *Curcuma* species along with others of the Zingiberaceae display diversity in habitat, ethnobotanical use, and morphology (Syamkumar and Sasikumar, 2007). Detailed knowledge about genetic relationships between wild and cultivated species of *Curcuma* will enhance the utilization value of wild species for any future study. *Curcuma* molecular biology studies refer to a few isozyme-based characterizations of germplasm accessions/species (Shamina et al., 1998; Apavatjrut et al., 1999; Paisooksantivatana et al., 2001) and analyses using ISSR, RAPD, and AFLP molecular markers. Previous results showed that RAPD, ISSR, and AFLP are very powerful methods for characterizing genetic relationships between species of *Curcuma*. However, ISSR (98.55%) marker demonstrated a different polymorphic capability compared to RAPD (93.22%) and AFLP (97.27%) and was found to be most informative in characterizing closely related *Curcuma* species from northeast India (Das et al., 2011). Yang and co-workers (1994) also found that ISSR assay can provide more informative data than other techniques. Syamkumar and Sasikumar (2007) showed that of eight ISSR primers, six (75%) gave 100% polymorphic bands among the 15 species of *Curcuma* and two primers namely $(TCC)_5$ AG and $(GACA)_3$ showed 70 and 90.91% polymorphism, respectively. Different hierarchical positions of the five *Curcuma* varieties in the dendrograms showed that genomes of each variety are not exactly the same. Also the dendrogram tree indicated that Chiang Mai Pink, Sweet Pink, Chiang Mai Red, and Kimono Pink varieties may have a common ancestor, which is different from that of the Doi Tung 554 variety. Group constellations were also independently developed by using principal component analysis (PCA) to verify grouping obtained through the dendrogram. PCA further helped in depicting the variability among the species in the three-dimensional modes. The first two PCA axes of the 5 varieties of *C. alismatifolia* are shown in Figure 3. PCA divided the varieties into three groups, a result similar to the dendrogram derived by UPGMA. Three principal components with eigenvalues greater than unity extracted a cumulative of 81.2% of the variance in 5 varieties of *C. alismatifolia*. For these, the three axes had eigenvalues of 58.1, 12.2, and 10.9%, respectively. PCA showed the three-dimensional relationships that describe portions of the genetic variance in a data set for *Curcuma* varieties.

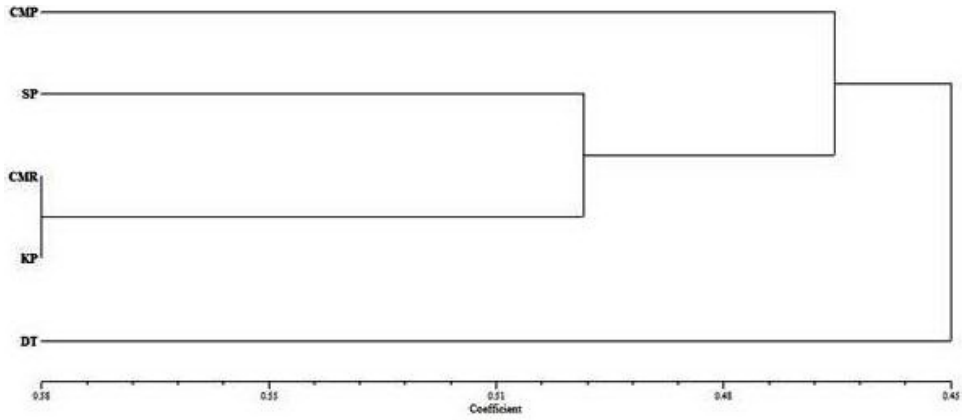

Figure 2. Dendrogram demonstrating the relationships among the 5 *Curcuma alismatifolia* varieties based on ISSRs.

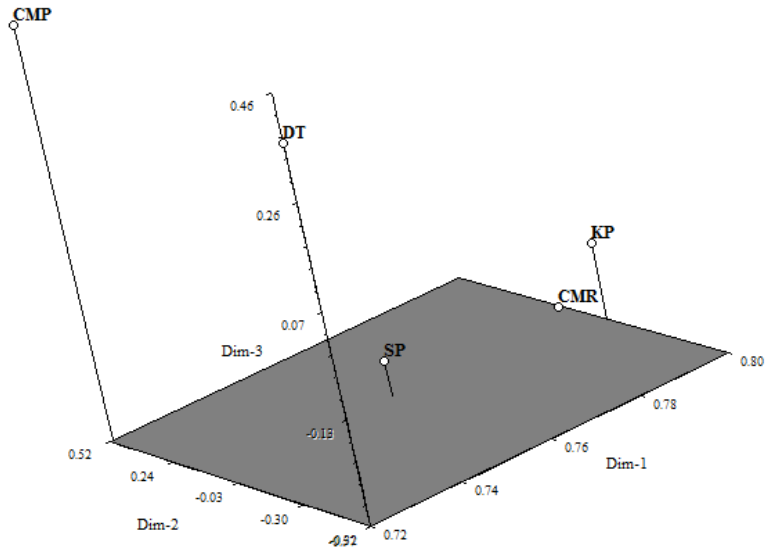

Figure 3. Principal component analysis map for ISSR markers of the 5 varieties of *Curcuma alismatifolia*.

CONCLUSION

This is the first report on the assessment of genetic variation in *C. alsmatifolia* varieties using molecular markers. In this study, cluster analysis based on ISSR markers divided the varieties into three distinct groups. The Chiang Mai Red and Kimono Pink varieties showed maximum similarity between them (JSI = 0.58), whereas the maximum dissimilarity belonged to Chiang Mai Pink, showing JSI = 0.40 with Doi tung 554 variety. The present investigation clearly demonstrated that the five *Curcuma* varieties could be distinguished by these ISSR primers, showing a high level of polymorphism with the ISSR technique, which

suggests that this marker amplification technique can be a useful and serve as a potentially powerful tool for genotyping studies in *Curcuma*.

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