GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Assessing the genetic relationships of *Curcuma alismatifolia* varieties using simple sequence repeat markers

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Genet. Mol. Res. 13 (3): 7339-7346 (2014)
Received June 28, 2013
Accepted July 5, 2014
Published September 5, 2014
DOI http://dx.doi.org/10.4238/2014.September.5.12

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 (SSRs) to elucidate genetic variation and relationships between five varieties of *Curcuma* (*Curcuma alismatifolia*) cultivated in Malaysia. Of the primers tested, 8 (of 17) SSR primers were selected for their reproducibility and high rates of polymorphism. The number of presumed alleles revealed by the SSR analysis ranged from two to six alleles, with a mean value of 3.25 alleles per locus. The values of H_O and H_E ranged from 0 to 0.8 (mean value of 0.2) and 0.1837 to 0.7755 (mean value of 0.5102), respectively. Eight SSR primers yielded 26 total amplified fragments and revealed high rates of polymorphism among the varieties studied. The polymorphic information content varied from 0.26 to 0.73. Dice's similarity coefficient was calculated for all pairwise comparisons and used to construct an unweighted pair group method with arithmetic average (UPGMA) dendrogram. Similarity coefficient values

from 0.2105 to 0.6667 (with an average of 0.4386) were found among the five varieties examined. A cluster analysis of data using a UPGMA algorithm divided the five varieties/hybrids into 2 groups.

Key words: *Curcuma alismatifolia*; Genetic relationships; Molecular markers; Simple sequence repeats

INTRODUCTION

Curcuma alismatifolia is a member of the Zingiberaceae family, which originates from the tropical and subtropical areas of northern Thailand and Cambodia (Apavatjrut et al., 1996). *C. alismatifolia* has recently become popular in the ornamental flower market because of their colorful, stiff, and long-lasting inflorescences, with pink-purplish coma bracts in the upper part and green coma bracts in the lower part. Small true flowers bear inside the bracts (Khuankaew et al., 2010). Traditional methods based on phenotypic observations for identifying *Curcuma* varieties in *Curcuma* spp. are slow and have limitations. However, new methods based on studies of DNA variations to assess genetic relationships or diversity among varieties can accelerate ornamental plant breeding programs. Molecular markers have become useful for breeding and cultivar development in many crops (Syamkumar and Sasikumar, 2007). Among the molecular markers available, microsatellites or simple sequence repeats (SSR), which are tandem repeats of 1-6 nucleotide long DNA motifs, have gained considerable importance in plant genetics and breeding because of their multi-allelic nature, codominant inheritance, high abundance, extensive genome coverage, reproducibility, and discriminatory power (Kalia et al., 2011). A variety of molecular marker techniques have been developed for measuring genetic variability/similarity in ornamental plants, such as random amplified polymorphic DNA (RAPD; *Anthurium andraeanum* and Chrysanthemum) (Nowbuth et al., 2005; Lapitan et al., 2007; Barakat et al., 2010), ISSR (Rose) (Jabbarzadeh et al., 2010), and SSR (*Prunus mume* and *Nelumbonucifera*) (Tian et al., 2008; Hayashi et al., 2008). Moreover, several molecular studies have employed various DNA markers in *Curcuma* spp. Apavatjrut et al. (1999) used isozyme markers to resolve the taxonomic confusion in the genus *Curcuma*. Sigrist et al. (2009) developed and characterized microsatellite markers (SSR) for *Curcuma longa* L. Das et al. (2011) used 12 RAPD, 19 ISSR, and 4 amplified fragment length polymorphism (AFLP) primers as molecular genetic fingerprints of 9 *Curcuma* spp. from northeast India, which were developed using polymerase chain reaction (PCR)-based markers. Taheri et al. (2012) used 16 ISSR primers to assess the genetic relationships of five *C. alismatifolia* varieties. They showed that the ISSR markers are more discriminating than those of RAPD and AFLP for evaluating the genetic diversity/relationships among *Curcuma* spp.

MATERIAL AND METHODS

Source of plant materials

The current study was conducted at the genetic and plant breeding laboratory in the 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 the *Curcuma* Nursery (Ubonrat) in Doisaket District, Chiang Mai 50220, Thailand.

Total genomic DNA extraction

Fully opened, fresh, and tender leaves of 25 samples of five varieties/hybrids of *C. alismatifolia* were used for the isolation of DNA. Approximately 0.1 g young leaf tissue was ground in liquid nitrogen into a fine powder and transferred to 2-mL microcentrifuge tubes. The genomic DNA was isolated using 800 µL modified cetyltrimethylammonium bromide (CTAB) extraction buffer (Doyle and Doyle, 1987). The extraction buffer was as follows: 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 mixture was incubated at 65°C for 1 h. The extraction was followed by the addition of 600 µL chloroform/isoamyl alcohol [24:1 (v/v)], and the resultant supernatants were transferred to new microcentrifuge tubes. Ethanol (95%) was used to precipitate nucleic acids, and the samples were kept at -20°C overnight. The pellet obtained was dissolved in a Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH = 8.0, and 1 mM EDTA, pH = 8.0). Coprecipitated RNA was removed by digestion with RNase. The concentration and purity of isolated DNA was determined using the NanoDrop 2000 (Thermo Fisher Scientific Inc.), and the quality was verified by electrophoresis on 0.8% agarose gel.

PCR amplification and product electrophoresis

PCR amplification was carried out for eight SSR primers in a 25-µL reaction volume. The PCR reaction contained 70 ng/µL genomic DNA, 2X DreamTaq™ Green PCR Master Mix (Fermentas, International Inc.), and 0.4 µM forward and reverse primers. Amplification was performed in a thermal cycler (Bio-Rad Laboratories, Inc.) for a total of 40 cycles after an initial denaturation of the template DNA at 94°C for 3 min. This was followed by 10 cycles of 94°C for 40 s, a touch-down one-degree decrement for 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, a final annealing temperature for 30 s and 72°C for 1 min, and a final extension of 72°C for 10 min. The amplification products were analyzed on 4% metaphor gel with a 50-bp DNA ladder (Ready-to-use). The gel was stained with Midori green, visualized under ultraviolet light, and photographed by a gel documentation system (ChemilImager™ Gel Doc., Alpha Innotech Corporation, San Jose, CA, USA).

Data scoring and analysis

In the case of SSR, each band was considered a separate putative locus. Only clear, unambiguous, and reproducible bands were considered for data analysis. Each band was considered a single locus. The presence or absence of each single fragment was coded by 1 or 0, respectively, and scored for a binary data matrix. The binary data matrix was entered into the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc 2.10e) (Rohlf, 2002). The level of similarity among species was established as the percentages of polymorphic bands and a matrix of genetic similarity compiled using Dice's similarity index (Dice, 1945). 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 (SHAN) routine in the NTSYSpc program, thus representing the genetic relationship among five *Curcuma* varieties. Measurement of diversity, including H_O, H_E (Nei, 1973), N_A, the effective number of alleles (N_E), Shannon's information

index (I), and the percentage of polymorphic bands (PPBs), were estimated using the POPGENE 1.31 software (Yeh et al., 1997). It was assumed that the gene frequency within a population was in Hardy-Weinberg equilibrium. To compare the efficiency of the primers, the polymorphic information content (PIC) as a marker discrimination power was calculated according to Cordeiro et al. (2000) using a PIC calculator (http://www.liv.ac.uk/~kempsj/pic.html). The calculation was based on the number of alleles per locus.

RESULTS AND DISCUSSION

SSR amplification

Of the 17 primer pairs, 9 showed monomorphic products. The remaining 8 primer pairs resulted in polymorphic and readable PCR products (Figure 1A-D). Table 1 summarizes the results obtained based on the analysis of five *Curcuma* varieties/hybrids using the polymorphic SSR loci. The N_A varied widely among these loci. The N_A ranged from 2 (clon04, clon09, clon11, and clon14) to 6 (clon12), with an average value of 3.25 per locus. On a per locus basis, these numbers were smaller than the average values of 6.6 and 6.7 (ranging from 2 to 11) alleles per locus for various classes of microsatellites reported by Sigrist et al. (2009).

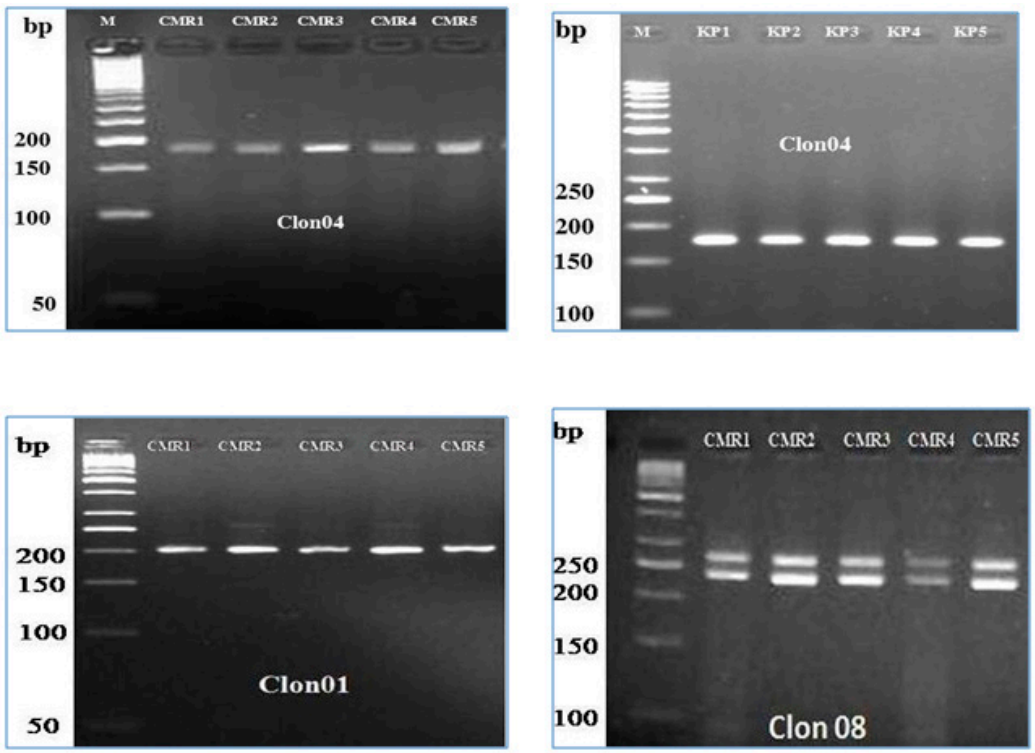

Figure 1. Banding pattern of different *Curcuma alismatifolia* varieties using simple sequence repeat (SSR) markers. *Lane M* = Marker; CMR = Chiang Mai Red; KP = Kimono Pink.

Table 1. SSR polymorphic primer sequences used for analysis of five varieties of *Curcuma alismatifolia* with N_A, percentage of polymorphism, product size, number of effective alleles, Nei's (1973) gene diversity, Shannon's index, and polymorphism information content (PIC).

Locus	Reference	Primer sequence (5´–3´)	Repeat motif	N_A	Range size (bp)	Tm (°C)	Ta (°C)	H_E	H_o	N_E	I	PIC
Clon01	Sigrist et al. (2009)	F: ACTGGACTGTCCGAGAGCAT R: TCGTTTAGCGACAACGGATT	$(TA)_6TTG(TC)_{16}$	4	194-212	60.7	68-58	0.7347	0.0000	3.5714	1.3322	0.67
Clon04	Sigrist et al. (2009)	F: TAAATTTGCGAAGGCAATCC R: CCGCAGAGGAATTTGAAGAG	$(TATAG)_2(AG)_{17}$	2	179-200	58.3	65-55	0.1837	0.2000	1.2195	0.3251	0.41
Clon08	Sigrist et al. (2009)	F: CCGGTGAGGGTGATATCTTG R: AAGCTCAAGCTCAAGCCAAT	$(GT)_{10}$	4	245-274	60.7	68-58	0.6531	0.8000	2.7778	1.1683	0.73
Clon09	Sigrist et al. (2009)	F: GGAGGAGGCAGTTGATTTGT R: GCTTTGGTGGCTAGAGATGC	$(AC)_{14}$	2	182-188	60.4	67-57	0.3265	0.0000	1.4706	0.5004	0.26
Clon10	Sigrist et al. (2009)	F: GTGGGAATTGGATTGCTCTC R: GAGAACTCCCCATGCTTCAG	$(GT)_7$	4	204-232	60.7	68-58	0.5918	0.2000	2.3810	1.0889	0.53
Clon11	Sigrist et al. (2009)	F: GGGCTTTGTTTAGTTGTCGTG R: CAGGAATGAAGTCGGCAAC	$(AGA)_8$	2	163-167	60.7	68-58	0.4898	0.0000	1.9231	0.6730	0.36
Clon12	Sigrist et al. (2009)	F: GATTGGATCACATGGTGTGC R: TGGGTTGATGGTTTCTCTGTT	$(CT)_{20}$	6	195-231	59.0	66-76	0.7755	0.4000	4.1667	1.6094	0.73
Clon14	Sigrist et al. (2009)	F: TCACGTCGAGGGGTTCCTACT R: GAGAGCTGATCGCAAAAACC	$(CTT)_7$	2	175-181	60.7	68-58	0.3265	0.0000	1.4706	0.5004	0.50
Total				26								
Mean				3.25				0.5102 ± 0.2146	0.2000 ± 0.2828	2.3726 ± 1.0676	0.8997 ± 0.4628	0.52

N_E = effective number of alleles; h = Nei's gene diversity; I = Shannon's information index; PIC = polymorphic information content.

The average N_A per locus obtained in the study was also smaller than that reported in previous studies using other types of markers such as RAPD, ISSR, and AFLP (Syamkumar and Sasykumar, 2007; Das et al., 2011), and allozymes (Paisooksantivatana et al., 2001). The N_E per locus ranged from 1.2 (clon04) to 3.5 (clon01), with an average of 2.3. The difference between the average N_A and N_E was due to the uneven frequency of each allele (Liao et al., 2011). PIC provides an estimate of the discriminatory power of a marker to differentiate genotypes based on both the N_A expressed and their relative frequencies (Nagl et al., 2011). The PIC value, which measures allele diversity and frequency among varieties, also varied from one locus to another. The PIC value was 0.52 per marker and ranged from 0.26 (clon09) to 0.73 (clon08 and clon12), indicating that most loci were highly polymorphic and informative. The genetic diversity of each SSR locus appeared to be associated with the N_A detected per locus. The smaller the PIC value of a locus, the fewer number of alleles detected. This pattern observed was consistent with that reported by Sigrist et al. (2009). Using Shannon's diversity index, an overall genetic diversity of 0.8997 was obtained from the analysis, indicating a relatively high level of genetic variation among studied varieties. The higher value of I represents the effectiveness of microsatellite loci to detect variation (Babaei et al., 2012). In this study, loci amplifying dinucleotide repeat motifs were found to be more polymorphic, with an average value of 5 alleles, than those with trinucleotide or tetra nucleotide repeat motifs, which both yielded an average value of two alleles. Among the loci with perfect or compound dinucleotide repeat motifs, the marker with the CT repeat motif showed the greatest variability. These results suggest that the total repeat count of SSR loci is associated with N_A (Lapitan et al., 2007). In this study, the greater the repeat number in the microsatellite DNA, the greater the N_A identified. The estimates of genetic similarity ranged from 21% for the most distant varieties (i.e., Sweet Pink and Kimono Pink) to 0.67% between Chiang Mai Red and Chiang Mai Pink (Table 2).

Table 2. Genetic similarity indices between each pair of the five *Curcuma* varieties (*C. alismatifolia*) based on SSR fragment analysis.

Varieties	Chiang Mai Pink	Sweet Pink	Doi Tung 554	Chiang Mai Red
Sweet Pink	0.6000			
Doi Tung 554	0.5263	0.2857		
Chiang Mai Red	0.6667	0.5000	0.4211	
Kimono Pink	0.3529	0.2105	0.3333	0.2353

Clustering of *Curcuma* varieties

Genetic similarity values among the *Curcuma* varieties led to the construction of a dendrogram (Figure 2). The UPGMA dendrogram showed two main clusters at a coefficient level of 0.38. The first one included only the Kimono Pink variety and yielded a Dice's similarity index of 0.28 in comparison to other varieties, while the second included the other four varieties. In the second cluster, there were two subclusters. The first subcluster included Doi Tung 554; Sweet Pink, Chiang Mai Red, and Chiang Mai pink were assigned to the second subcluster. A comparison of Chiang Mai Red and Chiang Mai Pink yielded the maximum observed similarity value (0.67 DSI).

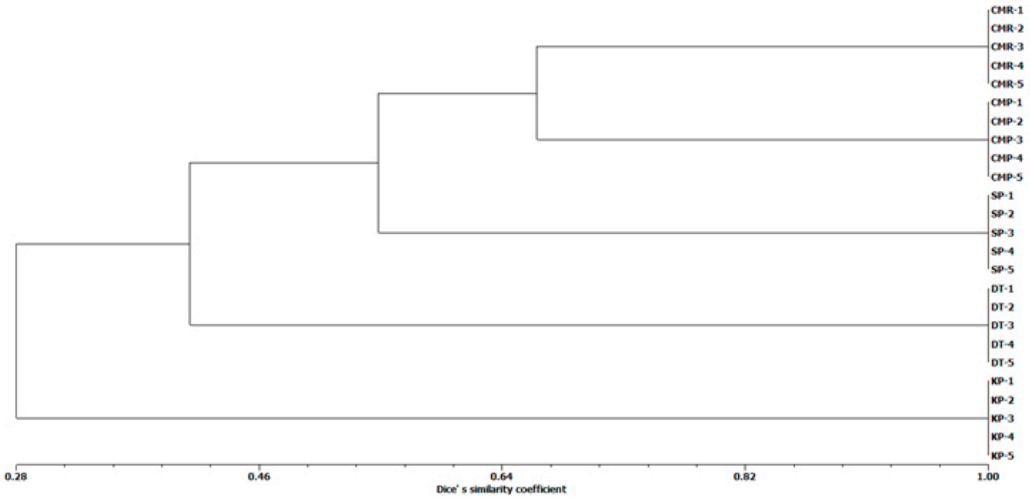

Figure 2. Dendrogram demonstrating the relationships among the 5 *Curcuma alismatifolia* varieties based on SSRs.

CONCLUSION

In summary, this study provided an overview of the genetic relationship of the *C. alismatifolia* varieties/hybrids. The use of SSR markers in genetic relationship analyses enabled the varieties to be grouped according to their genetic similarities/distances. Although there have been previously published reports on the use of other molecular marker techniques, such as RAPD, AFLP, and ISSR, for analyzing various species of *Curcuma*, the codominant nature of SSRs allows them to be used as a powerful tool for assessing the genetic diversities/relationships of the varieties under study. Additionally, the results obtained from this study confirm the complete similarity of different samples from each variety, and these results would be useful for improved management and identification of *C. alismatifolia* varieties for future breeding programs such as mutation breeding.

ACKNOWLEDGMENTS

Research supported by the fundamental research grant scheme (FRGS) under the Ministry of Higher Education in Malaysia.

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