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Research Communication

Use of Rice SSR Markers as RAPD Markers for Genetic Diversity Analysis in *Zingiberaceae*

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Species of *Zingiberaceae* display a diversity in habitat, ethnobotanical use and morphology. However, little is known about the genetic relationships among taxa and genetic diversity, primarily due to the lack of suitable molecular markers. We tested the cross-amplification potential of microsatellite markers among taxa to identify a larger number of genetic markers. To assess the applicability of rice microsatellite markers to the *Zingiberaceae*, we tested 12 microsatellite markers for 14 genotypes from three genera of this family: *Zingiber*, *Alpinia* and *Curcuma*. The origin of the genotypes was diverse, covering eight Asian countries. Four microsatellite primer sets failed to amplify fragments in all genotypes studied, whereas the other primer sets amplified all the genotypes. Among the 141 bands, that could be scored, 140 (99.5%) were polymorphic. On the average, each microsatellite primer set amplified 17.6 DNA fragments. In general, amplified fragments were larger than the original rice fragments including the microsatellite region, although in some cases, the amplified bands were similar in size. Though sequence analysis of these bands confirmed the absence of target repeat motif, amplification of a large number of polymorphic bands provided the basis to perform an analysis of genetic diversity. Primers could generate enough polymorphism for possible use in diversity studies, based on provisional multivariate analyses such as cluster analysis and principal component analysis (PCA). The whole set of genotypes based on molecular data was classified into four clusters after cluster analysis. Genotypes from the *Curcuma* and *Alpinia* genera were grouped into clusters I and II, respectively. Clusters III and IV comprised genotypes from the genus *Zingiber*. PCA led to a similar classification. The high polymorphism documented in the present study indicated that the rice microsatellite primers were useful for genetic diversity studies among genera in the family *Zingiberaceae*.

Key Words: genetic diversity, ginger, galangal, turmeric, SSR, *Zingiberaceae*.

Introduction

Ginger (*Zingiber* spp.), turmeric (*Curcuma* spp.) and galangal (*Alpinia* spp.) are important crop plants of the *Zingiberaceae*, a large family consisting of 53 genera and more than 1200 species found mainly in Asia, Africa and the Pacific Islands (Kress *et al.* 2002). These three genera have been cultivated for millennia in both China and India, mainly for use as spices (Foster 2000). Ginger is also a major ingredient in Indian traditional medicine (Rai *et al.* 1997). However, the genetic diversity and relatedness among the species in these groups are not well documented and few studies have been reported so far (Nayak *et al.* 2005, Saritnum *et al.* 2005). One of the major reasons is the lack of appropriate genetic markers and limited interest in underutilized taxa in academic research.

Microsatellites or simple sequence repeats (SSRs), which are genetic markers that identify alleles with high reliability and reproducibility (Gutierrez *et al.* 2005), are widely used in taxonomy and diversity studies. SSRs appear to be ubiquitous in higher organisms, although their frequency varies among species. They are generally abundant, dispersed throughout the genome, and also show higher levels of polymorphism than other genetic markers. Furthermore,

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they are codominantly inherited and suitable for automation, which are additional advantages over other types of molecular markers (Holten 2001). Major constraints on the use of SSRs are the cost and effort required for their development that have restricted their use to only a few agriculturally important crops (Gutierrez *et al.* 2005). The sequences flanking specific microsatellite loci in a genome are considered to be conserved within species, across species in a genus, and perhaps even across related genera (Varshney *et al.* 2002). Comparative genetics has revealed that gene content and order are highly conserved among closely related species.

Although studies on genetic diversity as well as cross-amplification in major crop plants are well documented, few deal with minor and underutilized crop species such as ginger, turmeric and galangal. No SSR markers are currently available for these species, and direct development of markers from these species would be prohibitively expensive and therefore impractical. Therefore, it is important to conduct cross-amplification studies on these crop species. Taking account of the monocot model of rice and *Zingiberaceous* genotypes, we tested the efficiency and practicality of the use of SSR markers from distantly related taxa (rice) for analyzing the cross-amplification and genetic diversity of the members of the *Zingiberaceae* family. Our objectives were to evaluate the cross-amplification potential of rice SSR markers for *Zingiberaceae* species, confirm the presence or absence of repeat motifs in the candidate species, and analyze genetic diversity based on polymorphism.

Materials and Methods

Plant materials and DNA isolation

We used 14 genotypes representing five species in three genera of the family *Zingiberaceae*. The samples originated from different countries (Table 1), and rice genotype T65 was used as the control. Total DNA was extracted from fresh leaves, according to the protocol of Syamkumar *et al.* (2003) with slight modifications. It is strongly recommended to use fresh leaf samples and to conduct experiments immediately, because *Zingiberaceae* species contain secondary metabolites that could potentially hamper enzymatic reactions after long-term storage and oxidization. To extract DNA, mercaptoethanol (1%) was added to the buffer, and the working solution of DNA was adjusted to 25 ng/μl. Extracted DNA was used to study the cross-amplification potential of rice SSR markers for *Zingiberaceae* species.

PCR amplification and sequencing

In total, we used 12 rice SSR markers. Table 2 lists each marker, repeat type and length, size range, primer sequence and annealing temperature. The final volume of the reaction mixture used for PCR analysis was 20 μl and the solution contained 1× ExTaq buffer, dNTPs (0.5 mM each), 1 unit of Ex Taq polymerase (TaKaRa), 0.5 μM of each forward and reverse primer, and 25 ng of DNA template. Amplification was conducted in a thermal cycler (Mycycler, ver 1.065, BioRad) with a 5-min initial denaturation at 94°C, followed by 35 cycles at 94°C for 1 min, annealing for 1 min, and an extension at 72°C for 1.5 min. A final extension was performed at 72°C for 10 min, followed by cooling to 10°C. PCR products were electrophoresed using 8% polyacrylamide gels and ethidium bromide staining. PCR products were cloned into the pGEM-T vector (Promega) and sequenced using the primer set M-13 and the BigDye Terminator Cycle Sequencing Kit with an ABI-3777 sequencer (Applied Biosystems Inc., USA).

Statistical analysis

DNA fragments showing polymorphism were scored as present (1) or absent (0) and used for statistical analyses. Genetic similarities among the genotypes were determined based on the Jaccard (1908) coefficient. A dendrogram was then constructed using the unweighted pair-group method of the arithmetic average (UPGMA). Principal component analysis (PCA) was performed to reveal genetic similarity and diversity among the genotypes.

Table 1. List of genotypes in three genera of the family *Zingiberaceae*

S. No.	Accession No.	Common name	Botanical name	Origin
1	ZO 14-1	Ginger	*Zingiber officinale*	Myanmar (Originally introduced from Japan)
2	ZO 25-1	Ginger	*Zingiber officinale*	Myanmar
3	ZO 29-1	Ginger	*Zingiber officinale*	Malaysia
4	ZO 33-1	Ginger	*Zingiber officinale*	Thailand
5	ZO 63	Ginger	*Zingiber barbatum*	Myanmar
6	ZO 72-1	Ginger	*Zingiber officinale*	Malaysia
7	ZO 75-1	Ginger	*Zingiber officinale*	Taiwan
8	ZO 79	Ginger	*Zingiber officinale*	Bangladesh
9	ZO 80-2	Ginger	*Zingiber officinale*	China
10	ZO 81	Ginger	*Zingiber officinale*	Pakistan
11	ZO 18-1	Turmeric	*Curcuma amada*	Myanmar
12	ZO 23-1	Turmeric	*Curcuma amada*	Myanmar
13	ZO 49	Turmeric	*Curcuma amada*	Malaysia
14	ZO 74-1	Galangal	*Alpinia officinarum*	Thailand

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Table 2. List of rice SSR primers tested in *Zingiberaceae*

Primer	Repeat type and length	Size range (bp)	Sequence	Annealing temp. in °C
RM1	$ACAA(AG)_{26}CCAC$	113	GCGAAAACACAATGCAAAAA GCGTTGGTTGGACCTGAC	55
RM131	$(CT)_9$	209–217	TCCTCCCTCCCTTCGCCCACTG CGATGTTCGCCATGGCTGCTCC	60
RM125	$(GCT)_8$	124–136	ATCAGCAGCCATGGCAGCGACC AGGGGATCATGTGCCGAAGGCC	55
RM171	$(GATG)_5$	318–343	AACGCGAGGACACGTACTTAC ACGAGATACGTACGCCTTTG	55
RM154	$(GA)_{21}$	165–169	ACCCTCTCCGCCTCGCCTCCTC CTCCTCCTCCTGCGACCGCTCC	60
RM153	$(GAA)_9$	189–204	GCCTCGAGCATCATCATCAG ATCAACCTGCACTTGCCTGG	55
RM72	$(TAT)_5C(ATT)_{15}$	152–198	CCGGCGATAAAACAATGAG GCATCGGTCCTAACTAAGGG	55
RM287	$(GA)_{21}$	98–118	TTCCCTGTTAAGAGAGAAATC GTGTATTTGGTGAAAGCAAC	55
RM135	$(CGG)_{10}$	119–131	CTCTGTCTCCTCCCCCGCGTCG TCAGCTTCTGGCCGGCCTCCTC	63
RM190	$(CT)_{11}$	104–124	CTTTGTCTATCTCAAGACAC TTGCAGATGTTCTTCCTGATG	55
RM278	$(GC)_{17}$	131–146	GTAGTGAGCCTAACAATAATC TCAACTCAGCATCTCTGTCC	55
RM117	$(AG)_7$	203–207	CGATCCATTCCTGCTGCTCGCG CGCCCCCATGCATGAGAAGACG	60

Results and Discussion

Polymorphism and Cross-SSR amplification

We tested the effectiveness of using rice microsatellite markers in terms of their cross-amplification potential in three genera of the family *Zingiberaceae*. Of 12 SSR primer sets, eight yielded band amplification across all the three genera, whereas four primer sets did not amplify any DNA fragments, except in control rice (Table 3). The eight SSR primer sets yielded 141 fragments, that could be scored, and the number of amplified fragments per reaction ranged from 5 to 26. Of the 141 DNA fragments, 140 (99%) were polymorphic, and each primer set amplified an average of 17.6 fragments. Genotype ZO 80-2 showed the highest number of amplified fragments (64), while the genotype ZO-49, the lowest (33). The number of amplified bands averaged 1.7–10.6 in the genus *Zingiber*, 1.0–8.3 in the genus *Curcuma* and 2.0–9.0 in the genus *Alpinia* (Table 3 and Fig. 1). The average total number of amplified bands in *Zingiber*, *Curcuma* and *Alpinia* was 51.5, 35.7 and 40.0, respectively. In general, the amplified fragments were large, compared to the target bands amplified in rice. The high level of polymorphism indicated that the family *Zingiberaceae* is highly diversified. The generation of highly polymorphic bands by rice SSR primer sets demonstrated their potential for use as RAPD markers in *Zingiberaceous* genotypes. The efficiency of rice

Table 3. General profile of DNA amplification by eight rice SSR primers across 14 genotypes in three genera of the family *Zingiberaceae*

Rice SSR markers	Total bands	Polymorphic bands	*Zingiber*											*Curcuma*				*Alpinia*
			ZO 14-1	ZO 25-1	ZO 29-1	ZO 33-1	ZO 63	ZO 72-1	ZO 75-1	ZO 79	ZO 80-2	ZO 81	Aver.	ZO 18-1	ZO 23-1	ZO 49	Aver.	ZO 74-1
RM1	11	11 (100%)	0	3	2	2	5	0	2	2	4	3	**2.3**	0	2	1	**1.0**	2.0
RM131	24	24 (100%)	11	12	9	10	10	8	8	2	13	11	**9.4**	6	6	3	**5.0**	8.0
RM125	26	25 (96%)	10	9	12	10	11	10	10	12	13	9	**10.6**	9	7	9	**8.3**	6.0
RM171	5	5 (100%)	1	1	1	3	2	0	3	3	3	0	**1.7**	0	0	0	**0.0**	2.0
RM154	20	20 (100%)	9	7	12	9	5	7	7	11	11	9	**8.7**	9	6	7	**7.3**	6.0
RM153	16	16 (100%)	4	4	3	2	2	4	3	0	3	2	**2.7**	3	4	0	**2.3**	3.0
RM135	25	25 (100%)	10	10	12	11	12	10	11	10	11	3	**10.0**	7	5	4	**5.3**	9.0
RM117	14	14 (100%)	5	8	6	7	4	6	7	6	6	6	**6.1**	6	4	9	**6.3**	4.0
Total	**141**	**140**	**50**	**54**	**57**	**54**	**51**	**45**	**51**	**46**	**64**	**43**	**51.5**	**40**	**34**	**33**	**35.7**	**40.0**
Average	**17.6**	**17.5 (99.5%)**											**51.5**				**35.7**	**40.0**

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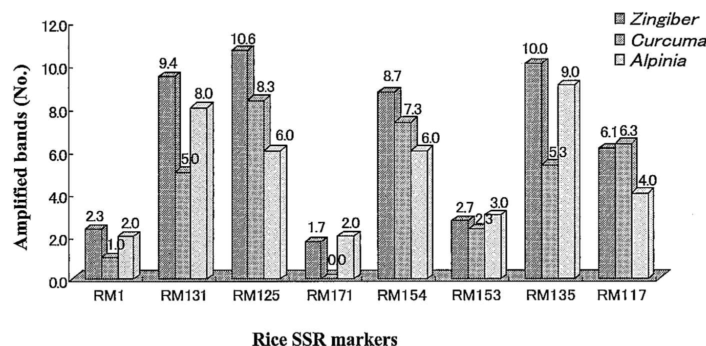

Fig. 1. Average number of bands amplified in *Zingiber*, *Curcuma* and *Alpinia* by eight rice SSR markers.

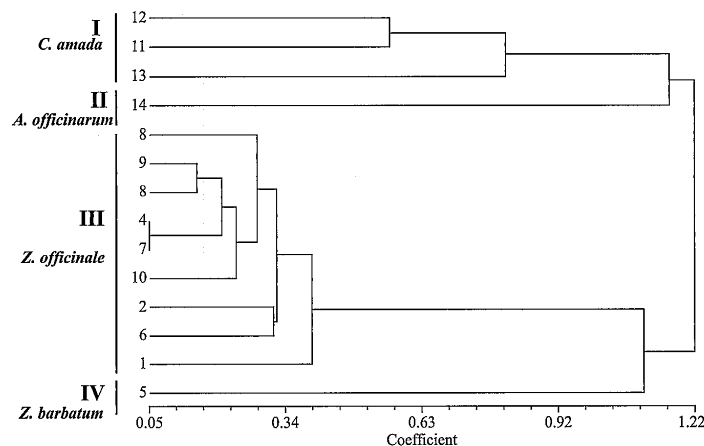

Fig. 2. Dendrogram based on eight rice SSR markers, indicating the genetic diversity among the members of the family *Zingiberaceae*. The horizontal axis shows the genetic similarity coefficient (Jaccard 1908). The number written against each bar denotes an individual accession and corresponds to those listed in Table 1.

SSRs as RAPD markers in the present study was appreciable, compared to genetic diversity studies based on RAPD markers in other species like vegetable water pepper (Yasuda and Yamaguchi 2005), upland cotton (Lu and Myers 2002), yam (Dansi *et al.* 2000) and wild, weedy and cultivated azuki beans (Mimura *et al.* 2000). Similarly, in genetic diversity analyses conducted in *Alpinia* (Saritnum *et al.* 2005) and *Zingiber* (Nayak *et al.* 2005), 8 and 20 RAPD primers generated 73 and 101 polymorphic bands, respectively, with an average number of polymorphic bands amplified by each RAPD marker ranging from 1 to 9. However, in the present study, the number of polymorphic bands generated by 8 rice SSR primer sets was 140 with an average of 17.5 bands per primer. The efficiency of the rice SSR markers in terms of amplification of a large number of fragments as well as average number of fragments per primer set was high, compared to RAPD markers (Saritnum *et al.* 2005, Nayak *et al.* 2005).

Amplification of several fragments with a large size reflected the low probability of the presence of target repeat motifs among the genotypes studied. However, in some genotypes, the size of the PCR products was in a similar range to that of rice fragments. Sequence analysis was, therefore, conducted to confirm the amplification of expected rice SSR markers for the target genotype. Candidate fragments were selected based on the band intensity as well as band size. Amplified PCR products with the corresponding forward and reverse microsatellite primers in one individual per species were used for sequencing. Sequence analysis revealed a general absence of repeat motifs in the amplified fragments. However, in rare cases, repeat motifs with few repeat numbers were identified. As the genotypes under study were distantly related, the absence of repeat motifs in the amplified fragments could be anticipated. However, in some sequenced fragments, sequence homology in genotypes belonging to *Zingiber*, *Curcuma* and *Alpinia* (data not shown) exceeded more than 95%.

Genetic diversity Analysis

Scoring of the polymorphic fragments was subjected to multivariate analyses. Dendrograms based on UPGMA analysis separated 14 genotypes into four clusters with a Jaccard's similarity coefficient of 0.05–1.22 (Fig. 2). Cluster I represented the genotypes in the genus *Curcuma*, and cluster II comprised the only representative genotype from the genus *Alpinia*. Within cluster I, ZO 18-1 and ZO 23-1 were closer to each other than to ZO-49. Clusters III and IV comprised genotypes in the genus *Zingiber*. Further within the genus *Zingiber*, genotypes representing *Z. officinale* were grouped together (Cluster III), whereas the genotype from *Z. barbatum* was classified separately (Cluster IV). As illustrated by the dendrogram, cluster III was the largest, and within this cluster, ZO 33-1 and ZO 75-1 were much closer to each other than to any other genotypes, whereas ZO-79 and ZO 14-1 were farther from each other than from any other genotypes. The results of the PCA were comparable to the cluster analysis showing a similar topology (data not shown). The present genetic diversity analysis based on molecular data depicted the high resolution power of rice SSR markers for *Zingiberaceae* members. The classification among different genera as well as among species within the genus *Zingiber* indicated that these markers were useful for studying the genetic diversity of the family *Zingiberaceae*. The grouping pattern reported in the present study supported the current taxonomic classification of the family. However, to confirm the wider usefulness of these SSRs as RAPD markers, similar studies on large populations of different genera and multiple species within each genus should be conducted.

In the present study, the genus *Zingiber* constituted a larger group than the *Curcuma* and *Alpinia* genera. As the genotypes clustered in this group represented a diverse geographical region, data were analyzed to determine the variation pattern within the genus. PCA enabled to classify these genotypes into two groups (Fig. 3). The contribution of PC1, PC2 and PC3 was 41.9%, 14.7% and 10.7%, respectively, and the cumulative contribution was 67.3%. In this scatter plot, *Z. barbatum* (ZO 63) again constituted a separate group, whereas within the second group representing *Z. officinale*,

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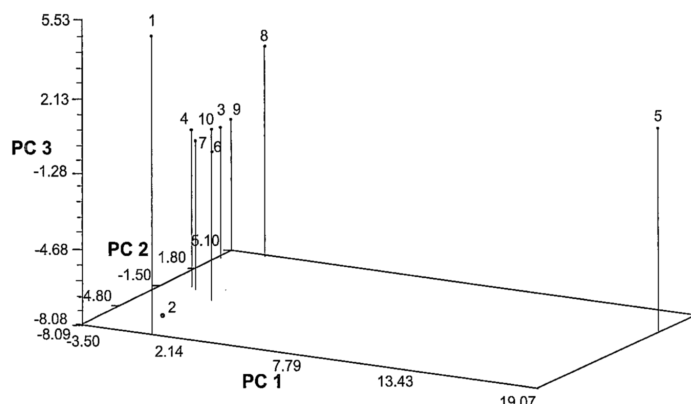

Fig. 3. PCA scatter plot of ten genotypes in the genus *Zingiber* based on eight rice SSR markers. The contribution of PC1, PC2 and PC3 was 41.9%, 14.7% and 10.7%, respectively. The cumulative contribution was 67.3%. The numbers plotted represent individual accessions and correspond to those listed in Table 1.

most genotypes were closer to each other. However, the genotypes ZO 14-1 and ZO 79 were only distantly related. Clustering patterns within the genus *Zingiber* did not reflect any relationship between genotypic variation and collection origin.

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