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Chromosome numbers, RAPD and ISSR profiles of six *Zingiber* species found in Manipur, India

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Abstract. *Bidyaleima L, Kishor R, Sharma GJ. 2019. Chromosome numbers, RAPD and ISSR profiles of six* Zingiber *species found in Manipur, India. Biodiversitas 20: 1389-1397.* The present investigation was done to assess the cytological information and genetic relationships among six species of *Zingiber*, viz., *Zingiber capitatum*, *Z. kangleipakense*, *Z. kerrii*, *Z. montanum*, *Z. officinale* and *Z. zerumbet* found in Manipur, North-East India. The somatic chromosome numbers observed were 2n = 22 for *Zingiber capitatum*, *Z. kerrii*, *Z. montanum*, *Z. officinale* and *Z. zerumbet*, whereas *Z. kangleipakense* showed tetraploidy with 2n = 44. The somatic chromosome numbers of *Zingiber kangleipakense* and *Z. kerrii* are reported for the first time. Randomly amplified polymorphic DNA (RAPD) and inter-specific sequence repeat (ISSR) molecular markers were employed to reveal the genetic relationships among the six species. The pair-wise Jaccard genetic similarity varied from 0.518 to 0.658 for RAPD and from 0.436 to 0.682 for ISSR data. Dendrogram derived from the combined data of RAPD and ISSR clustered the six members into two groups. The detected polymorphism level represents high genetic distance at the inter-species level, and introduces RAPD and ISSR as efficient markers for the assessment of genetic relatedness in *Zingiber*. Our results may provide useful information for application in breeding, conservation and utilization.

Keywords: Cytology, genetic relatedness, ISSR, RAPD, *Zingiber*

INTRODUCTION

The genus *Zingiber* Mill. consists of 100 to 150 species worldwide and their diversity and distributions are mainly concentrated in Thailand, China and the Indian Sub-continent (Wu and Larsen 2000; Triboun 2006). In India, the genus is represented by 20 species (Vasantha 2009). Sabu (2006) recorded 8 species from South India while Tripathi and Singh (2006) reported 7 species from North-East India. Manipur (NE India) lies within the Indo-Burmese mega-biodiversity 'hotspot' region and houses several wild and domesticated species of medicinal gingers. With a new species and three new additions to the *Zingiber* flora, so far eight species have been described from Manipur, *viz*., *Zingiber capitatum* Roxb., *Z. chrysanthum* Roscoe, *Z. kangleipakense* Kishor & Škorničk., *Z. kerrii* Craib., *Z. montanum* (J.König) Link ex A. Dietr., *Z. officinale* Roscoe, *Z. roseum* (Roxb.) Roscoe and *Z. zerumbet* (Linn.) Roscoe ex Sm. (Deb 1961; Sharma et al. 2011; Kishor and Škorničková 2013; Thongam et al. 2013; Devi et al. 2016, 2017).

Excepting a few domesticated and cultivated ones most of the *Zingiber* species are less investigated and understood taxonomically and remain as under-utilized crops. A review of published works revealed that only 17 species of *Zingiber* have been investigated for their cytology till now (Table 1). Most of the species of this genus are highly consistent with a somatic chromosome number of 2n = 22 with a few exceptions. The established base number of *Zingiber* is x = 11 (Chakravorti 1948; Sato 1960; Mahanty 1970; Omanakumari and Mathew 1985).

PCR-based molecular markers are broadly applied for identification, population studies, phylogenetic evaluation and genetic linkage mapping in many plant species (Williams et al. 1990). Kress et al. (2002) proposed a new classification of Zingiberaceae based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *mat*K regions. They utilized 104 species under 41 genera representing all four tribes of the Zingiberaceae. The new classification of the Zingiberaceae recognized four sub-families and four tribes: Siphonochiloideae (Siphonochileae), Tamijioideae (Tamijieae), Alpinioideae (Alpinieae, Riedelieae), and Zingiberoideae (Zingibereae, Globbeae). Ngamriabsakul et al. (2003) also performed a phylogenetic analysis of the tribe *Zingibereae* (*Zingiberaceae*) using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA (*trn*L (UAA) 5′ exon to *trn*F (GAA) and concluded the tribe to be monophyletic with two major clades, the *Curcuma* clade, and the *Hedychium* clade. Relationships among 23 species of *Zingiber* were investigated using nuclear ribosomal DNA (ITS1, 5.8S, and ITS2) sequences (Theerakulpisut et al. 2012).

Interspecific DNA-based genetic relationships analyses of the genus *Zingiber* were carried out by various authors (Jiang et al. 2006; Jatoi et al. 2008; Vasantha 2009; Bua-in and Paisooksantivatana 2010; Ghosh et al. 2011; Mohanty et al. 2014; Siriluck et al. 2014). The most popular *Zingiber* species on which molecular markers tools were applied is

Zingiber officinale (Rai et al. 1997; Damayanthi 1998; Rout et al. 1998; Jatoi et al. 2008; Singh et al. 2013). Vasantha (2009) used the *rbc*L sequences for studying genetic relationships of nine South Indian *Zingiber* species. Rout et al. (1998) demonstrated that RAPD analysis can be applied to assess the genetic fidelity of micropropagated plants of *Zingiber officinale* derived in vitro on an industrial scale as part of crop improvement programs. All RAPD profiles from micropropagated plants were monomorphic and no variation was detected within the micropropagated plants. This method might be useful for monitoring the stability of in vitro germplasm collections and cryopreserved material. Intraspecific genetic variation of cultivated *Z. officinale* and its three wild congeners, *viz*., *Z. neesanum*, *Z. nimmonii* and *Z. zerumbet* from South India were assessed using AFLP (Kavitha et al. 2010). Jatoi et al. (2008) also studied genetic relationships among three *Zingiber* species, *viz*., *Z. officinale*, *Z. barbatum* and *Z. mioga* using RAPD.

As there has been constant addition of new members in this genus, it is imperative to fully understand its genetic resource, and hence to study them with parameters like cytology and molecular markers. The main objective of the present study, therefore, is to determine the somatic chromosome numbers of six *Zingiber* species found in Manipur and to establish their genetic relationships using RAPD and ISSR markers.

MATERIALS AND METHODS

Plant materials

Specimens of different *Zingiber* species were collected from various locations of Manipur (Table 2) and maintained in the Experimental Garden of the Department of Life Sciences, Manipur University. The biological material used for DNA analysis was harvested from the young shoots and leaves. For cytological analyses, roots were harvested and used.

Table 1. Chromosome numbers reported in the genus *Zingiber*

Taxa	2n	Reference
Z. capitatum Roxb.	22	Mandi (1990)
Z. capitatum Roxb. var. *elatum* (Roxb.)	22	Vasantha (2009)
Z. cernuum Dalzell	22	Joseph (1998), Vasantha (2009)
Z. clarkei King ex Baker	22	Holttum (1950)
Z. cylindricum Thwaites	22	Mohanty (1970)
Z. gramineum Noronha ex Blume	32	Etikawati and Setywan (2000)
Z. mioga (Thunb.) Roscoe	55	Morinaga et al. (1929), Sato (1948)
Z. montanum (J.König) Link ex A.Dietr.	22	Raghavan and Venkattasubban (1943), Chakravorti (1948), Mandi (1990), Vasantha (2009)
Syn. *Z. purpurium* Roscoe	22	Joseph (1998)
	32	Etikawati and Setywan (2000)
Z. neesanum (J. Graham) Ramamoorthy Syn. *Z. macrostachyum* Dalzell	22	Ramachandran (1969), Omanakumari and Mathew (1985), Joseph (1998), Vasantha (2009)
Z. nimmonii (J. Graham) Dalzell	22	Vasantha (2009)
Z. officinale Roscoe	22	Sugiura (1928), Raghavan and Venkattasubban (1943), Chakravorti (1948), Sharma and Bhattacharya (1959), Ramachandran (1969), Omanakumari and Mathew (1984), Joseph (1998), Mandi (1990), Eksomtramage (2002), Vasantha (2009), Daryono et al. (2012), Bhadra and Bandyopadhyay (2015)
	24	Chakraborti (1948), Sharma and Bhattacharya (1959), and Dhamayanthi and Zachariah (1998)
	32	Etikawati and Setywan (2000)
Z. officinale Rosc. var. *officinale*		
Z. officinale Rosc. var. *amarum*	30	Daryono et al. (2012)
Z. ottensii Valeton	22	Holttum (1950)
	32	Etikawati and Setywan (2000)
Z. roseum (Roxb.) Roscoe	22	Ramachandran (1969), Mandi (1990), Joseph (1998), Vasantha (2009)
Z. rubens Roxb.	22	Chakravorti (1948), Mandi (1990)
Z. spectabilis Griff.	22	Mohanty (1970)
Z. wightianum Thwaites	22	Chakravorti (1948), Ramachandran (1969), Omanakumari and Mathew (1984), Mandi (1990), Vasantha (2009)
Z. aff. *wrayi* Prain ex Ridl.	22	Eksomtramage (2002)
Z. zerumbet (L.) Roscoe ex Sm.	22	Chakravorti (1948), Ramachandran (1969), Omanakumari and Mathew (1984), Joseph (1998), Mandi (1990), Vasantha (2009), Bhadra and Bandyopadhyay (2015)
	32	Etikawati and Setywan (2000)

Table 2. Details of *Zingiber* species collected from different locations in Manipur, India

Taxa	Section	Collection site	Location	Altitude (m)
Zingiber capitatum Roxb.	*Dymczewiczia*	Wangoo, Kakching	24°23′51″N, 93°51′25″E	787
Z. kangleipakense Kishor & Škorničk.	*Cryptanthium* or *Pleuranthesis*	Canchipur, Imphal West	24°45′06″N, 93°55′39″E	779
Z. montanum (J. Köenig) Link ex. Dietr.	*Zingiber*	Thanga, Bishnupur	24°32′03″N, 93°49′55″E	809
Z. officinale Rosc.	*Zingiber* or *Dymczewiczia*	Lamsang, Imphal West	24°49′09″N, 93°52′18″E	789
Z. zerumbet (L.) Rosc. ex Sm.	*Zingiber*	Katomei, Senapati	25°16′50″N, 93°01′28″E	1127
Z. kerrii Craib.	*Zingiber*	Wangkhem, Thoubal	24°40′00″N, 93°01′33″E	786

Cytological analysis

Actively growing healthy smooth white root-tips from rhizomes at about 2 cm in length were harvested and pre-treated with saturated solutions of p-dichlorobenzene for 4 h at 12°C. After thoroughly washing three times in distilled water, the root-tips were re-suspended in freshly prepared Carnoy's fluid and stored at 12°C for 24 h. The root-tips were subjected to enzyme treatment (2% cellulase and 2% pectinase) for a duration of 40-55 minutes which gave the best results (Song et al. 1988). After enzyme treatment, root-tips were thoroughly washed. For hydrolysis, the time period of treatment of 1 N HCl varied from 6-8 minutes. Squash preparations were made in acetocarmine and duration of staining varied from 6-24 hours. After staining, the root-tips were squashed in 45% acetic acid in clean grease–free slides. Chromosome numbers of 20 cells of each species were determined at well-spread metaphase stage. The squashed root-tips were examined from temporary preparations under a light microscope (Leitz DIALUX 22) and photographs were taken with LEICA D-LUX 3 digital camera.

DNA extraction

Genomic DNA was extracted from young shoots and leaves of the respective *Zingiber* species. Total DNA was isolated using 2% (w/v) CTAB with a slight modification of Doyle and Doyle (1987) DNA extraction protocol and purified after RNase treatment. The extraction buffer contained 2% CTAB, 1.42 mM NaCl (pH 8.0), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 4% (w/v) PVP and 2% (v/v) β–mercaptoethanol. Quantification was done using Biophotometer (Eppendorf, Germany) and quality was checked on 1.2% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) at 80 V for 2 h.

RAPD analysis

Out of 30 primers, 24 were selected based on their amplification pattern and reproducibility for RAPD analysis following the protocols developed by Williams et al. (1990). RAPD primers were synthesized at Xcleris Genomics Prime X Company (Ahmedabad, India) by providing sequences of Operon Technologies (USA) and University of British Colombia (Canada). PCR amplification was carried out at 94°C for 1 min, primer annealing for 1 min at 53°C, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The reaction mixture (25µl) contained 10 ng genomic DNA, 1X reaction buffer, 200 µM of dNTPs (Genie, Merck Specialities Private Limited, Mumbai, India), 0.4 µM of each primer and 1 Unit of Taq DNA Polymerase (Sigma-Aldrich, USA). The reactions were carried out in a DNA thermocycler (Gene Amp PCR System 9700, Applied Biosystems, USA). The amplification products were analyzed on 1.8% agarose gel with a 100-bp DNA ladder (Genie, Merck Specialities Pvt. Ltd., Mumbai, India) and photographed using a Gel Documentation System (Vilber Lourmat, France). All PCR results were tested for reproducibility for at least three times.

ISSR analysis

For ISSR analysis, ten primers from Sigma-Genosys, USA were screened for preliminary analysis and eight primers gave reproducible bands. PCR amplification was carried out at 94°C for 4 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min at different temperatures depending on primers (Table 5), extension at 72°C for 1 min and a final extension at 72°C for 10 min. Only reproducible products were taken into account for further data analysis.

Data scoring and analysis

Only clear, reproducible and unambiguous bands were considered for data analysis. Data were scored as '1' for presence and '0' for absence. The percentage polymorphism, polymorphism information content (PIC), effective multiplex ratio (EMR), resolving power (Rp) and marker index (MI) were calculated. Percentage polymorphism was calculated as the percentage of polymorphic loci from total loci obtained per primer. Polymorphism information content (PIC) values of individual primers were calculated based on the formula PIC = 2 x F (1-F) (Anderson et al. 1993). Marker index, a product of information content and EMR were calculated following (Powell et al. 1996). Rp of each primer combination was calculated according to (Prevost and Wilkinson 1999). The Jaccard's similarity index was calculated using NTSYS-PC 2.02e (Applied Biostatistics Inc., Setauket, NY, USA) to compute pairwise Jaccard's similarity coefficient (Jaccard, 1908) and this similarity matrix was used in cluster analysis using an unweighted pair group method with arithmetic averages (UPGMA) and sequential, agglomerative, hierarchical and nested (SAHN) clustering algorithm to obtain a dendrogram (Rohlf 1998).

RESULTS AND DISCUSSION

Chromosome count

Chromosome numbers of all six species were determined. The results with 2n = 2x = 22 were observed in *Zingiber capitatum*, *Z. montanum*, *Z. officinale*, *Z. zerumbet*, *Z. kerrii*, and 2n = 4x = 44 in *Z. kangleipakense* (Figure 1).

RAPD analysis

For RAPD analysis of the six species of *Zingiber*, 24 random decamer primers were chosen after an initial screening of 30 primers and they amplified across all the species studied with reproducible results (Table 3). A total of 515 amplified fragments were resolved by the 24 primers used, of which 468 were polymorphic. Each primer, thus, produced on an average 21.46 amplified fragments, the minimum being 9 with the primer UBC-05 and the maximum being 34 with the primer OPA-09 (Figure 2.A). Size of the amplification products ranged from 130 to 1800 bp. Polymorphic percentage of these RAPD primers varied from 70.6% in OPD-05 to 100% in OPA-01 and OPC-07, with an average of 89.9%. The polymorphism information content (PIC) of the primers ranges from 0.310 in OPD-05 to 0.429 in OPQ-06, with an average of 0.367. The resolving power (Rp) of the primers varied from 0.449 in OPQ-05 to 0.688 in UBC-01, with an average of 0.585. The average marker index (MI) of the primers was 0.331with minimum value of 0.219 in OPD-05 to the maximum value of 0.423 in OPC-07.The dendrogram obtained was based on the UPGMA analysis of the binary RAPD data (Figure 3.A). The use of Jaccard's (J) coefficient to estimate genetic relatedness among the six species gave similarity values ranging from 0.50 to 0.62 (Table 4). The first cluster (Cluster I) consisted of the three species – *Zingiber capitatum* (Z1), *Z. montanum* (Z3) and *Z. kerrii* (Z6). The highest similarity was noticed between *Z. capitatum* (Z1) and *Z. montanum* (Z3) with similarity coefficient value of 0.62. The second cluster (Cluster II) had three species – *Z. kangleipakense* (Z2), *Z. zerumbet* (Z5) and *Z. officinale* (Z4).

ISSR analysis

A total of 8 primers out of initially screened 10 were chosen for final amplification reactions. The total number of DNA fragments produced by these primers was 119 with 110 of them being polymorphic (Table 5). A minimum of 6 (UBC-824) to a maximum of 20 [UBC-808 (Figure 2.F) and UBC-862)] fragments were produced individually by these primers with an average of 14.88 fragments per primer. The size of the amplified DNA fragments varied from 260-1750 bp. Percentage polymorphism of the primers varied from 87.5% in UBC-861 to 100% in UBC-820 and UBC-824 and the average was found to be 93.02%. These primers vary in resolving power between 0.389 (UBC-824) and 0.648 (UBC-820) with an average of 0.544 (Table 6). The marker index of the primers varied from 0.280 in UBC-861 to 0.404 in UBC-820 with an average of 0.334.

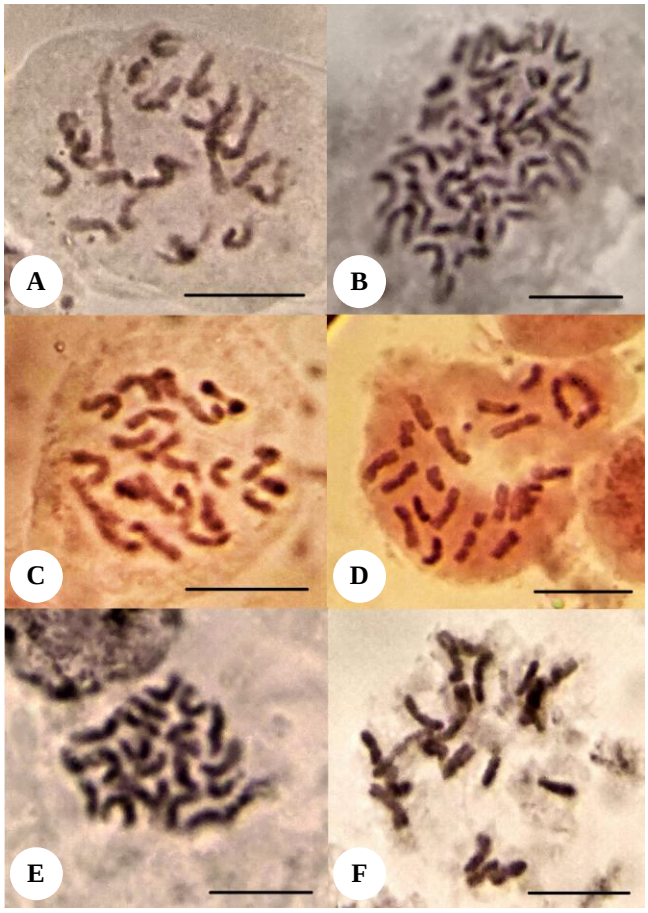

Figure 1. Somatic metaphase chromosomes in six *Zingiber* species. A) *Zingiber capitatum* (2n = 22); B) *Z. kangleipakense* (2n = 44); C) *Z. montanum* (2n = 22); D) *Z. officinale* (2n = 22). E) *Z. zerumbet* (2n = 22) and F) *Z. kerrii* (2n = 22) Bar = 5µm.

The dendrogram that was produced by UPGMA analysis showed a variation of similarity coefficient from 0.47 to 0.68 (Figure 3.B). Cluster I consisted of *Zingiber capitatum* (Z1), *Z. montanum* (Z3), *Z. kerrii* (Z6), *Z. officinale* (Z4) and *Z. zerumbet* (Z5). *Zingiber capitatum* (Z1) and *Z. montanum* (Z3) showed a similarity value of 0.68. Cluster II separated out *Z. kangleipakense* (Z2).

Combined RAPD and ISSR analysis

A dendrogram based on UPGMA analysis was generated using the binary data of RAPD and ISSR together (Figure 3.C). The dendrogram revealed the Jaccard's similarity coefficient varying from 0.50 to 0.63 (Table 7). Cluster I consisted of *Zingiber capitatum* (Z1), *Z. montanum* (Z3), *Z. kerrii* (Z6) and *Z. officinale* (Z4). *Z. capitatum* (Z1) and *Z. montanum* (Z3) showed the maximum similarity among the six species studied with a similarity value of 0.63. *Zingiber kangleipakense* (Z2) and *Z. zerumbet* (Z5) together formed cluster II.

Table 3. Results of RAPD analysis and RAPD oligonucleotide primers information

RAPD primer	Sequence (5′–3′)	Annealing T°C	NTL[a]	NPL[b]	P%[c]	PIC[d]	R_P[e]	MI[f]	Approx. Product size
OPA-01	CAGGCCCTTC	53	27	27	100.0	0.374	0.568	0.374	200-1420
OPA-04	AATCGGGCTG	53	27	25	92.6	0.399	0.668	0.370	200-1700
OPA-09	GGGTAACGCC	53	34	33	97.1	0.382	0.588	0.370	200-1800
OPA-11	CAATCGCCGT	53	30	28	93.3	0.363	0.556	0.339	170-1710
OPA-15	TTCCGAACCC	53	26	25	96.1	0.399	0.654	0.384	180-1750
OPC-05	GATGACCGCC	53	22	20	90.9	0.353	0.545	0.321	280-1505
OPC-07	GATGACCGCC	53	24	24	100.0	0.423	0.680	0.423	220-1490
OPC-11	GTCCCGACGA	53	23	20	86.9	0.350	0.537	0.304	350-1550
OPD-03	AAAGCTGCGG	53	20	16	80.0	0.325	0.517	0.260	130-650
OPD-05	GTCGCCGTCA	53	17	12	70.6	0.310	0.490	0.219	300-830
OPD-08	TGAGCGGACA	53	18	15	83.3	0.352	0.592	0.293	210-700
OPD-11	GTGTGCCCCA	53	16	14	87.5	0.361	0.583	0.316	170-1000
OPQ-05	AGCGCCATTG	53	26	23	88.5	0.314	0.449	0.278	250-1500
OPQ-06	CCGCGTCTTG	53	18	17	94.4	0.429	0.685	0.405	310-850
OPU-05	TTGGCGGCCT	53	20	19	95.0	0.394	0.667	0.374	200-1350
OPU-16	CTGCGCTGGA	53	21	19	90.5	0.333	0.508	0.301	350-1410
UBC-01	CCTGGGCTTC	53	17	16	94.1	0.385	0.688	0.362	310-1430
UBC-02	CCTGGGCTTG	53	17	15	88.2	0.320	0.471	0.282	210-890
UBC-03	CCTGGGCTTA	53	15	13	86.7	0.355	0.578	0.308	230-1400
UBC-04	CCTGGGCTGG	53	24	21	87.5	0.368	0.598	0.322	190-900
UBC-05	CCGGCCTTAA	53	9	7	77.8	0.351	0.555	0.273	280-1100
UBC-06	CCGGCTGGAA	53	26	24	92.3	0.384	0.615	0.354	300-1700
UBC-09	GAGGGCGAGC	53	14	13	92.9	0.405	0.667	0.376	420-1500
UBC-12	GAGCTCGCGA	53	24	22	91.7	0.372	0.569	0.341	290-1500
Total			515	468					
Mean					89.9	0.367	0.585	0.331	

Note: [a] Number of total loci (NTL), [b]Number of polymorphic loci (NPL), [c] Polymorphism percentage (P%), [d] Polymorphism information content (PIC), [e] Resolving power (R_p) and [f] Marker index (MI)

Table 5. Results of ISSR analysis and ISSR oligonucleotide primers information

ISSR primer	Sequence (5′–3′)	Annealing T°C	NTL[a]	NPL[b]	P%[c]	PIC[d]	R_P[e]	MI[f]	Approx. Product size (bp)
UBC-808	$(AG)_8C$	42	20	18	90.0	0.389	0.633	0.350	260-980
UBC-815	$(CT)_8G$	40	12	11	91.7	0.352	0.556	0.323	500-1600
UBC-818	$(CA)_8G$	47	15	14	93.3	0.333	0.422	0.311	380-1750
UBC-820	$(GT)_8C$	46	18	18	100.0	0.404	0.648	0.404	320-1400
UBC-824	$(TC)_8G$	46	6	6	100.0	0.306	0.389	0.306	450-1500
UBC-827	$(AC)_8G$	50	12	11	91.7	0.389	0.611	0.357	320-1400
UBC-861	$(ACC)_6$	63	16	14	87.5	0.320	0.458	0.280	270-1200
UBC-862	$(AGC)_6$	63	20	18	90.0	0.378	0.633	0.340	390-1200
Total			119	110					
Mean					93.02	0.359	0.544	0.334	

Note: [a] Number of total loci (NTL), [b] Number of polymorphic loci (NPL), [c] Polymorphism percentage (P%), [d] Polymorphism information content (PIC), [e] Resolving power (R_p) and [f] Marker index (MI)

Table 4. Jaccard's similarity coefficient of six *Zingiber* species based on RAPD data analysis

	Z1	Z2	Z3	Z4	Z5	Z6
Z1	1.000					
Z2	0.518	1.000				
Z3	0.658	0.542	1.000			
Z4	0.569	0.550	0.565	1.000		
Z5	0.550	0.561	0.553	0.557	1.000	
Z6	0.565	0.546	0.600	0.522	0.565	1.000

Note: Z1: *Zingiber capitatum*, Z2: *Z. kangleipakense*, Z3: *Z. montanum*, Z4: *Z. officinale*, Z5: *Z. zerumbet* and Z6: *Z. kerrii*

Table 6. Jaccard's similarity coefficient of six *Zingiber* species based on ISSR data analysis

	Z1	Z2	Z3	Z4	Z5	Z6
Z1	1.000					
Z2	0.482	1.000				
Z3	0.682	0.436	1.000			
Z4	0.582	0.464	0.536	1.000		
Z5	0.527	0.482	0.500	0.491	1.000	
Z6	0.627	0.509	0.564	0.555	0.482	1.000

Note: Z1: *Zingiber capitatum*, Z2: *Z. kangleipakense*, Z3: *Z. montanum*, Z4: *Z. officinale*, Z5: *Z. zerumbet* and Z6: *Z. kerrii*

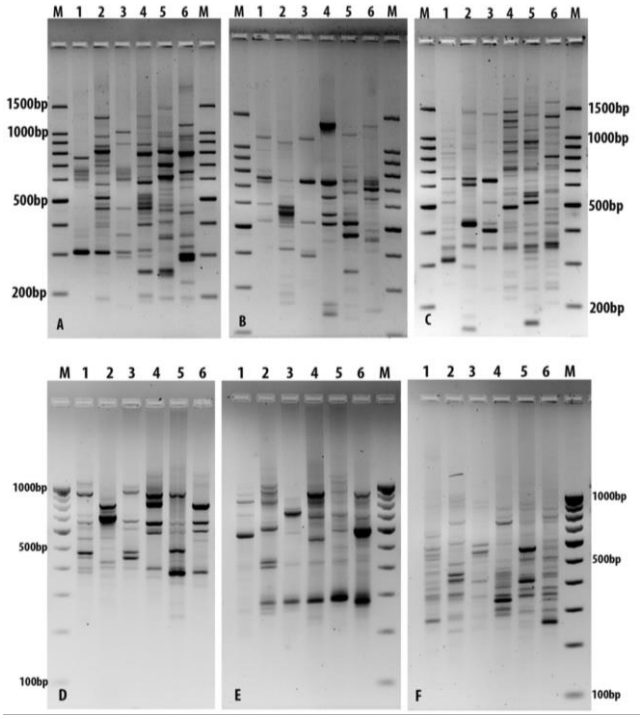

Figure 2. PCR products of genomic DNA from six *Zingiber* species with RAPD primers, (A) OPA-9, (B) OPU-16, (C) UBC-06 and ISSR primers, (D) UBC-862, (E) UBC-861, (F) UBC-808. Lane M: 100bp DNA Ladder, Lane 1: *Zingiber capitatum*, Lane 2: *Z. kangleipakense*, Lane 3: *Z. montanum*, Lane 4: *Z. officinale*, Lane 5: *Z. zerumbet* and Lane 6: *Z. kerrii*

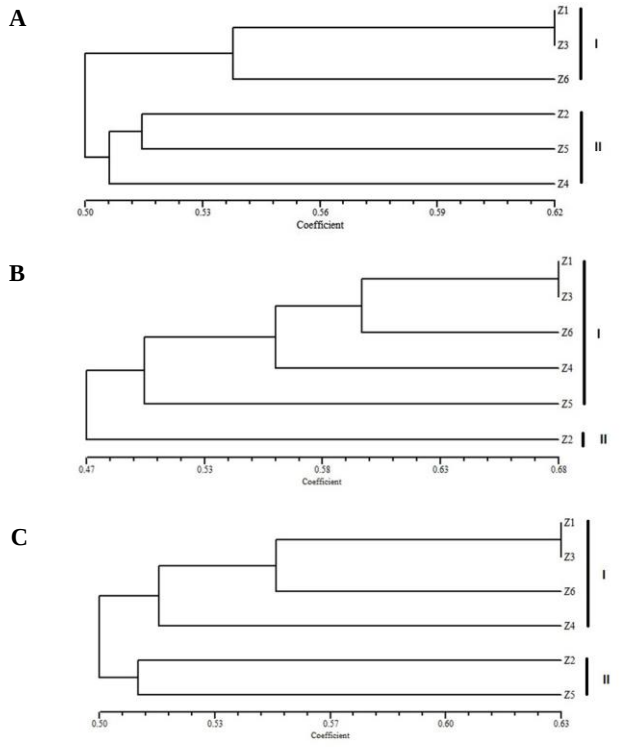

Figure 3. Dendrograms demonstrating the relationships among six *Zingiber* species based on (A) RAPD, (B) ISSR and (C) RAPD and ISSR. Z1: *Zingiber capitatum*, Z2: *Z. kangleipakense*, Z3: *Z. montanum*, Z4: *Z. officinale*, Z5: *Z. zerumbet* and Z6: *Z. kerrii*

Table 7. Jaccard's similarity coefficient of six *Zingiber* species based on combined (RAPD+ISSR) data analysis

	Z1	Z2	Z3	Z4	Z5	Z6
Z1	1.000					
Z2	0.472	1.000				
Z3	0.635	0.484	1.000			
Z4	0.536	0.497	0.524	1.000		
Z5	0.509	0.510	0.507	0.509	1.000	
Z6	0.542	0.502	0.561	0.490	0.514	1.000

Note: Z1: *Zingiber capitatum*, Z2: *Z. kangleipakense*, Z3: *Z. montanum*, Z4: *Z. officinale*, Z5: *Z. zerumbet* and Z6: *Z. kerrii*

Discussion

Manipur with its varied agro-climatic conditions, ranging from sub-tropics to sub-alpine, supports a rich diversity of ginger flora (Sharma et al. 2011). Here, we report a study of the relationships among six *Zingiber* species using cytological and molecular parameters. The six species used in this study belong to different sections based on inflorescence types. *Zingiber capitatum* is characterized by its terminal inflorescence (Sect. *Dymczewiczia*), green bracts with red margins and pale-yellow flowers which open in the evening. The inflorescences of *Z. kangleipakense* are generally borne directly from the rhizome resembling numerous species of section *Cryptanthium* but the inflorescence sometimes protrudes through the pseudostem, a characteristic known from section *Pleuranthesis.* For these reasons, it is difficult to place *Z. kangleipakense* unequivocally in a section (Kishor and Škorničková 2013). *Zingiber officinale* which normally develops radical, erect inflorescences (Sect. *Zingiber*), can also produce inflorescences apically on a leafy shoot (Sect. *Dymczewiczia*) in some rare instances (Triboun 2006). Theilade (1999) suggested that the genes determining the development of these two types of inflorescence may vary in their expression or that the habit of inflorescences may be triggered by environmental factors. The remaining three species, *viz.*, *Z. kerrii*, *Z. montanum* and *Z. zerumbet* belong to section *Zingiber*. Even though the number of species studied is small, they represent all the sectional classification based on inflorescence types.

The earliest chromosomal study of Zingiberaceae was done by Sugiura (1928) and reported the somatic chromosome number of *Zingiber officinale* to be 2n = 22. The genus *Zingiber* showed a constant somatic chromosome number of 2n = 22 and a very few reports of variations exist. In the present investigation chromosome complements of six *Zingiber* species have been established showing that five of them have 2n = 22 except *Z. kangleipakense* which has 2n = 44. The somatic numbers (2n = 22) found confirmed that the five *Zingiber* species are diploids. Raghavan and Venkatasubban (1943) investigated 25 taxa under seven genera of Zingiberaceae including three *Zingiber* species *Z. officinale*, *Z. cassumunar* and *Z. zerumbet* and found somatic chromosome 2n = 22 in all the species. They further claim that the chromosome morphology of *Z. zerumbet* and *Z. cassumunar* are almost identical whereas the chromosomes

of *Z. officinale* are different from the rest, not only in respect of their slender nature but in their morphology also. Ramachandran (1969) studied cytology of 27 species under 11 genera of Zingiberaceae including five species of *Zingiber*, *viz*, *Z. roseum*, *Z. wightianum*, *Z. zerumbet*, *Z. macrostachyum* and *Z. officinale* and reported somatic chromosome 2n = 22 in all the species. Omanakumari and Mathew (1984) carried out detailed karyomorphological study of four species of *Zingiber* from South India, *viz.*, *Z. officinale*, *Z. zerumbet*, *Z. wightianum* and *Z. macrostachyum*. Karyomorphological data indicate that except *Z. officinale*, where it is relatively symmetrical, all the other three species are moderately asymmetrical. Mahanty (1970) determined chromosome number of 33 species of Zingiberaceae including two *Zingiber* species namely, *Z. spectabile* and *Z. cylindricum* with somatic number 2n = 22 in each species. Joseph (1998) worked out karyomorphological details of six species, *viz.*, *Zingiber cernuum*, *Z. neesanuum*, *Z. officinale*, *Z. purpureum*, *Z. roseum*, *Z. zerumbet* with 2n = 22 and reported uniformity in structure with minute structural alterations. Eksomtramage et al. (2002) determined chromosome number of 22 species (with three *Zingiber* species) belonging to 10 genera of Zingiberaceae distributed in Thailand and showed a uniform chromosome number 2n = 22 in all three *Zingiber* species. Vasantha (2009) carried out a cytological study of *Zingiber* in South India, represented by nine species, *viz*., *Z. capitatum* var. *elatum*, *Z. cernuum*, *Z. montanum*, *Z. neesanum*, *Z. nimmonii*, *Z. officinale*, *Z. roseum*, *Z. wightianum* and *Z. zerumbet* and observed 2n = 22 in all the species.

Tetraploidy in the genus *Zingiber* is reported here for the first time with Z. *kangleipakense* having 2n = 44. Variation in chromosome numbers from 2n = 22 has been reported in some *Zingiber* species like *Z. moiga* (Morinaga et al. 1929 and Sato 1948) and *Z. officinale* (Chakraborti 1948; Sharma and Bhattacharya 1959; Dhamayanthi and Zachariah 1998; Etikawati and Setyawan 2000; Daryono et al. 2012). It was suggested by Chakravorti (1948) that the somatic chromosome number 55 of *Z. mioga* indicates pentaploidy with a basic number of 11. The sporadic occurrence of 2n = 24 in some cultivars of ginger apart from constant metaphase chromosome number of 2n = 22 might be due to abnormal spindle function in metaphase during clonal multiplication or due to the residual effects of mutagens during synthetic establishment of the ginger cultivars (Rai et al. 1997).

RAPD and ISSR markers have been applied to assess the genetic diversity at the inter-specific level among six *Zingiber* species. Dendrograms based on UPGMA analyses reveal two clusters for both RAPD and ISSR. Cluster analyses based on RAPD and ISSR combination also group the six *Zingiber* species into two clusters. The first cluster includes *Z. capitatum* and *Z. montanum* in all the three studied marker systems. Based on morphological traits, we expect high genetic distance between *Z. capitatum* and other species. Most of the RAPD and ISSR primers used in the present study were able to successfully amplify for orchids (Kishor and Devi 2009) and *Acorus calamus* (Devi et al. 2018). Based on the values of marker index and resolving powers, they concluded that the ISSR markers were more efficient than the RAPDs. Variations in DNA sequences lead to polymorphism and greater polymorphism are indicative of greater genetic diversity. The highest similarity between *Z. capitatum* and *Z. montanum* has been obtained in ISSR. RAPD analysis also reveals the high genetic similarity between *Z. capitatum* and *Z. montanum*. These results indicate that the two species are closely related. The variation between RAPD and ISSR may be due to the fact that PCR profiles are amplified from different non-repetitive and repetitive regions of the genome in the two marker systems (Thormann et al. 1994). RAPD, ISSR and their combined analyses show *Z. capitatum* and *Z. kangleipakense* as the most divergent ones revealing the existence of consistency between the two marker systems for estimation of genetic variation in *Zingiber*. Our data reveal the similarity between *Z. capitatum* and *Z. kangleipakense* based on RAPD, ISSR and combined analyses respectively.

The genus *Zingiber* is considered as a strongly monophyletic group by Kress et al. (2002) and Theerakulpisut et al. (2012) who performed phylogenetic analyses of the genus Zingiber based on ITS data. Jatoi et al. (2008) investigated the genetic variability of *Zingiber officinale* from ex-situ gene bank, farm and rural markets, and the genetic relationships of three *Zingiber* species, *viz*., *Z. officinale*, *Z. barbatum* and *Z. moiga* using SSR markers. RAPD marker is an effective tool for investigating genetic diversity in *Zingiber* both at interspecific and intraspecific levels (Bua-in and Paisooksantivatana, 2010). Mohanty et al. (2014) studied genetic diversity and gene differentiation among ten species of Zingiberaceae (*Zingiber officinale*, *Z. rubens*, *Z. zerumbet*, *Z. chrysanthum*, *Z. clarkei*, *Z. montanum* (Syn. *Cassumunar*), *Curcuma longa*, *C. amada*, *C. aromatica* and *C. caesia*) from Eastern India and concluded that within the *Zingiber* genus *Z. montanum* was isolated from the rest of the *Zingiber* species. They found that analysis of combined data of RAPD, ISSR and SSR markers resulted in better distinction of individual species.

Jiang et al. (2006) investigated the interspecific differences based on the metabolic profiling and phylogenetic analysis by utilizing *rpsl*6 and *trn*L-F regions amongst *Zingiber officinale*, *Z. mioga*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet*, and *Alpinia galanga* as the outgroup. The phylogenetic trees generated revealed identical structure with only observed difference, i.e., *Z. zerumbet* to be more closely related to *Z. officinale* based on molecular data, whereas *Z. montanum* was more closely related to *Z. officinale* based on the chemical data. A similar relationship is observed in our RAPD and ISSR data analyses, showing *Z. zerumbet* more closely related to *Z. officinale* (Figure 3.A-B).

Ghosh et al. (2011) utilized amplified fragment length polymorphism (AFLP) to produce DNA fingerprints for three *Zingiber* species, *viz*., *Z. officinale*, *Z. montanum* and *Z. zerumbet*, and suggested that *Z. montanum* and *Z. zerumbet* are phylogenetically closer to each other than to *Z. officinale*. However, from our study, it is observed that *Z. montanum* and *Z. officinale* are phylogenetically closer to each other as evidenced by the combined analysis of

RAPD and ISSR (Fig. 3C). Siriluck et al. (2014) used ISSR markers for identification of twenty-four species of Zingiberaceae (including five *Zingber* species, *viz*., *Z. officinale*, *Z. kerrii*, *Z. mekongense*, *Z. ottensii* and *Z. montanum*) in Thailand. Their result confirmed that *Z. officinale*, *Z. kerrii*, *Z. mekongense*, and *Z. montanum* were able to be classified into the same group with 80% genetic relationship. Our results are in agreement with their report that the *Z. montanum* and *Z. kerrii* are closely grouped. RAPD, ISSR and combined markers show similar groupings.

Our data based on two marker systems reveal the existence of low genetic similarity at the inter-specific level among the studied species. Thirty-two selected markers introduce sufficient overview of the relationships among the six *Zingiber* species and reveal that PCR based fingerprinting techniques are informative for estimating the extent of genetic diversity as well as determining the pattern of genetic relationships. Data suggest that both DNA markers are effective and reliable molecular markers for accurate assessment of genetic variation. Analyses reveal *Z. kangleipakense* as the most divergent one with tetraploid somatic chromosome complement. These findings may be beneficial in germplasm management activities in maximizing the genetic diversity of *Zingiber*.

In conclusion, cytological investigations of the six *Zingiber* species provided the interesting chromosome complements. Particularly, the record finding of a tetraploid species, *Z. kangleikapakense* is indeed extremely significant. RAPD and ISSR markers have introduced sufficient perspective of the genetic relationships among the six *Zingiber* species and revealed that PCR-based fingerprinting techniques are informative enough for estimating the extent of genetic diversity as well as determining the pattern of genetic relationships.

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